NOAA TECHNICAL MEMORANDUM NMFS-SEFSC-329

BIOMEDICAL TEST MATERIALS PROGRAM: ANALYTICAL METHODS FOR THE QUALITY ASSURANCE OF FISH OIL: SECOND EDITION

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U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Science Center
Charleston Laboratory
P. O. Box 12607
Charleston, SC 29412-0607

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Gloria T. Seaborn
Janet A. Gooch
Frances M. Van Dolah
Sylvia B. Galloway

Editors

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U.S. DEPARTMENT OF COMMERCE
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National Oceanic and Atmospheric Administration
D. James Baker, Undersecretary and Administrator

National Marine Fisheries Service
Nancy A. Foster, Acting Assistant Administrator for Fisheries

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National Technical Information Service
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Charleston Laboratory
P. O. Box 12607
Charleston, South Carolina 29412-0607

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INTRODUCTION

The compilation of methods contained in this manual represents the standard methods utilized by the National Marine Fisheries Service (NMFS) FISH OIL BIOMEDICAL TEST MATERIALS (BTM) PROGRAM to conduct quality assurance and quality control. The BTM Program was initiated in 1986 by the signing of a memorandum of understanding (MOU) between the National Oceanic and Atmospheric Administration (NOAA) and the National Institutes of Health (NIH)/Alcohol, Drug Abuse and Mental Health Administration (ADAMHA). Under the MOU it was agreed that the Charleston Laboratory of NMFS would provide a long-term consistent supply of test materials in order to facilitate the evaluation of the role of omega-3 fatty acids in health and disease. A sub-committee of the Nutrition Coordinating Committee of NIH, the Fish Oil Test Materials Advisory Committee (FOTMAC), provides the review and approval mechanism for the distribution of quality assured/quality controlled test materials to researchers. The applicants are researchers who are funded by NIH, ADAMHA, and other research organizations.

The unique contribution of seafood lipids (oils) to human health began to unfold with the publication of a series of Danish studies on the low incidence of heart disease in the Greenland Eskimos. A number of subsequent studies in this and other countries have led to the hypothesis that increased consumption of seafood or fish oils rich in omega-3 polyunsaturated fatty acids (PUFA) can have direct and positive influence in preventing or ameliorating many degenerative disease processes. At a conference ('Health Effects of Polyunsaturated Fatty Acids in Seafoods', Washington DC, 1985) of leading researchers in these areas, it was concluded that a significant limitation in the research was the lack of adequate supplies of quality assured test materials of consistent composition to explore the many research frontiers identified by the conferees. The BTM Program was designed to respond to this need for reliable test materials for the period of years necessary to complete the research.

The Charleston Laboratory currently produces vacuum-deodorized menhaden oil (VDMO) and >85% omega-3 (n-3) PUFA ethyl ester concentrates, provided in bulk form or gelatin encapsulated. Purified ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are also produced and provided in small quantities. A quality assurance report accompanies each lot of test materials shipped from the laboratory detailing the chemical composition, oxidation and degradation products, and low levels of contaminants that may be present in the products. This information is used by the investigators to alert them of any minor components which may possess physiological activity in their particular experimental system affecting the interpretation of their results.

Many of the methods used are official methods of the Association of Official Analytical Chemists' (AOAC) or American Oil Chemists' Society (AOCS). Many of the official methods were developed for the analysis of vegetable oils and have been modified for analysis of fish oil. In some cases, current technology far exceeds the official method, and the program has utilized the newer technique. All methods are described in detail, and include information on results of collaborative efforts using the method.

The manual contains nine sections including the general introduction. Each section of the manual deals with specific types of analyses: lipid characterization, sterols, fatty acid

oxidation products, organics, metals, moisture, sensory attributes, and bacteria. Each method is described using the following outline: introduction, principle, apparatus, reagents, preparation of standards and samples, determination, calculation, precision, notes, and references.

Many of the methods included in this manual require the use of hazardous chemicals, compressed gases, and a variety of analytical equipment. Users of this manual are encouraged to consult safety data sheets, equipment maunfacturers instructions, and general laboratory safety guidelines before proceding with any of these methods.

The editors wish to acknowledge the following contributors for their invaluable assistance in compiling the methods presented in this volume; Jeanne D. Joseph, Cheryl R. Sivertsen, Patsy P. Bell, Vijay A. Koli, Gregory B. Mitchum, Yvonne Taylor, and Laura Webster. We also wish to acknowledge Janette Smith for assistance in preparing figures and Meryl Reese for clerical assistance.

The mention of trade names is for informational purposes and is not intended as an endorsement of the product(s) by the National Marine Fisheries Service.

1.0 LIPID CHARACTERIZATION

1.1 LIPID CLASS PROFILE

INTRODUCTION

Thin layer chromatography (TLC) is used as a semi-quantitative technique to monitor product composition through all phases of production and to assure purity prior to product distribution. TLC and gas chromatography are the chromatographic methods most frequently used for lipid separation (1). TLC is particularly effective for the separation of intact complex lipids and neutral lipids (2,3). Although no official AOAC method exists for the examination of lipid composition by TLC, many standard methods employ TLC as a means of isolation and purification of sample components for subsequent analysis.

PRINCIPLE

Fats, oils, and other neutral lipids are separated by adsorption chromatography on glass plates pre-coated with a 250μm analytical layer of silica gel G. Lipid samples and purified standards, usually in chloroform, are applied as discrete spots, 1.5-2.0 cm from the lower edge of the plate by means of a microsyringe or other suitable applicator. The application solvent is evaporated and the plate is placed in a developing tank that contains the eluting solvent (approximately 1 cm in depth). As the eluting solvent ascends by capillary action, neutral lipid components are separated according to their affinity for the adsorbent silica gel. Polar lipids remain, as a single spot, at the point of application. The separated components are visualized after treatment with a suitable indicator. These components are tentatively identified by comparison of migration distances with those of authentic standards. Size and intensity of spots is the basis for estimated amounts of each component.

APPARATUS

- Analytical balance
- Fume hood
- TLC plates, 20 cm x 20 cm, precoated silica gel G, with preadsorbent layer (J.T. Baker Si250F PA or equivalent) stored in a desiccator.
- Syringe for sample application (Hamilton #701N or equivalent)
- Desiccator for storing plates (Alltech Assoc., Inc)
- Glass chromatography tank with glass cover (Supelco)

- Adsorbent paper for lining tanks (Alltech Assoc., Inc)
- Spotting guide (Supelco)
- Drying oven (GCA/Precision Scientific)
- Chromist sprayer (Gelman Sciences)
- TLC spray box (Alltech Assoc., Inc)
- TLC plate scriber (Alltech Assoc., Inc)
- Amber glass vials, 1.5 ml, with screw-caps fitted with teflon-lined septa (Supelco)

REAGENTS

- Hexane:diethyl ether:glacial acetic acid (80:20:1 v/v/v) (ACS reagent grade)
- Chloroform (ACS reagent grade)
- Phosphomolybdic acid (reagent grade), 5% (w/v) in 95% ethanol, stored in amber bottle
- Ethanol, 95% (reagent grade)
- Mixed TLC standards (steryl esters, methyl esters, triacylglycerol, free fatty acid, cholesterol, mono- and di-acylglycerol, Nu Chek Prep catalog Nos. 18-1-A and 18-4-A), 99+% purity, in sealed ampules.

PREPARATION OF STANDARDS & SAMPLES

1. Standards: Standards, in glass ampules, should be stored at -20°C. Prepare chloroform solutions of the mixed standards with concentrations of 20-25 µg/µl. Store in nitrogen-purged amber vials, fitted with crimp or screw caps with teflon-lined septa, at -20°C.

2. Samples: Dissolve each lipid sample in chloroform to give a concentration of 20-25 µg lipid/µl and store in nitrogen-purged amber vials, fitted with crimp or screw caps with teflon-lined septa.

DETERMINATION

1. Line the chromatography tank on three sides with adsorbent paper. Add 100 ml solvent, cover and allow 1-2 h for equilibration. A weight may be placed on the glass lid to prevent evaporation of solvent. All development, solvent evaporation, iodine exposure and indicating spray procedures should be conducted in a fume hood.

2. Remove TLC plate from desiccator immediately prior to spotting and place on a clean, dust-free surface. Disposable latex gloves should be worn when handling plates to prevent contamination of the plate with skin lipids.

3. If desired, the plate can be channeled using a plate scriber.

4. Position the spotting guide so that the sample may be applied to the plate approximately 0.5 cm below the upper edge of the preadsorbent area (approximately 1.5-2.0 cm. from the lower edge of the plate) and centered in the channel. Make a horizontal score in the coating material approximately 2 cm from the top of the plate. (See Figure 1.1-1)

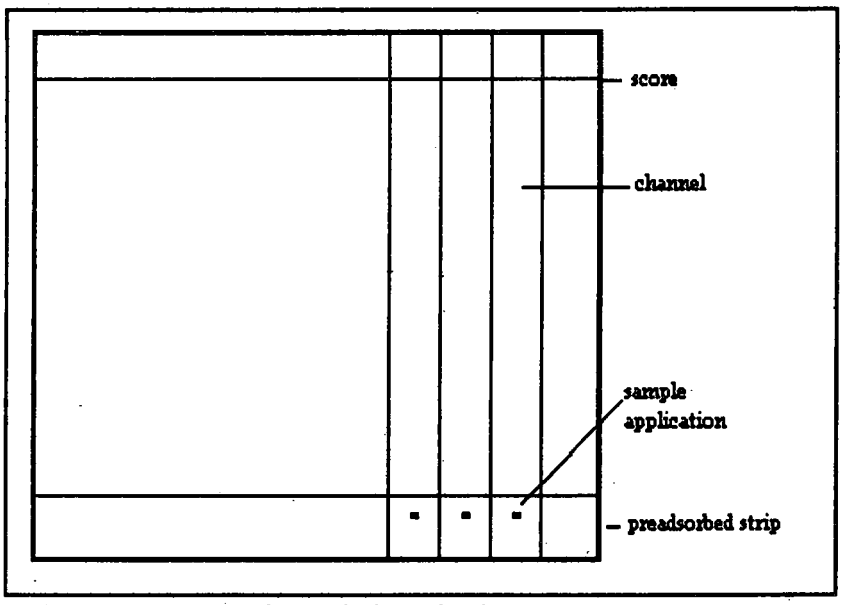

FIGURE 1.1-1. Channeled TLC Plate.

5. Spot a 10 and 20 µl volume of each sample solution and a 10 µl volume of each of the two standards. Allow the chloroform to evaporate by passing clean, dry nitrogen over the surface of the plate.

6. Place the plate in the developing tank and allow the solvent to ascend to the score line (30-40 min). Remove the plate from the tank and allow the solvent to evaporate.

7. Place the plate in a securely covered chamber, containing iodine crystals, until

component spots are clearly defined. The spots generally disappear on exposure to atmosphere, therefore, observations are best made before the plate is removed from the iodine chamber. For the purpose of permanent records the plate may be covered with a clean 20 x 20 cm glass plate and photocopied or photographed immediately.

8. More intense color for specific components can often be achieved by spraying the plate evenly with phosphomolybdic acid.

9. After spraying, transfer the plate to 110°C drying oven for 5 min, or until best spot definition is achieved.

CALCULATION

TLC is used by the BTM Program to semi-quantitatively estimate lipid composition. Lipid components are identified by comparison with migration distance of standards spotted on the same plate. Iodine forms dark brown spots for all unsaturated and some saturated lipids. Approximately 1 μg of an unsaturated component can be detected. (3). Steryl esters, methyl/ethyl esters, triacylglycerols, free fatty acids, tocopherols, mono and diacylglycerols, and total polar components, of both marine and vegetable oils, are readily visualized with iodine. Cholesterol and tocopherol spots are intensified with phosphomolybdic acid spray. This often provides a means of distinguishing cholesterol spots from those of diacylglycerols. These two components are not well separated in the solvent system specified in this method. Typical separations are llustrated in Figure 1.1-2. Plates may be photographed or photocopied for permanent records.

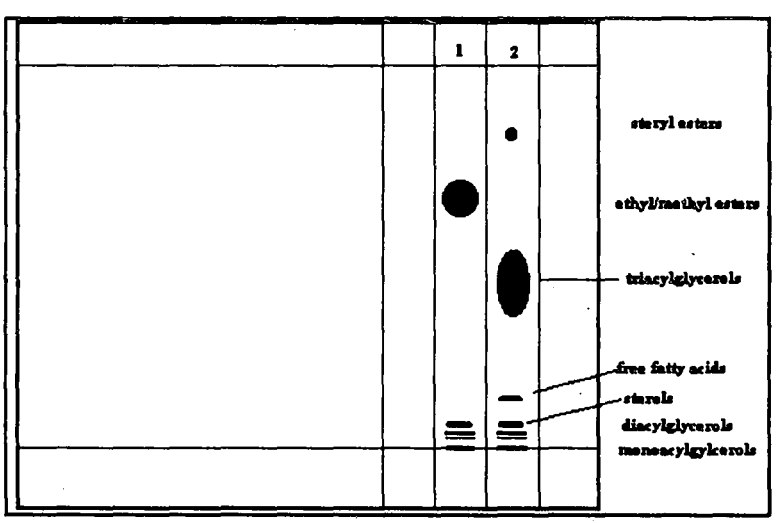

FIGURE 1.1-2. Illustrated Separation for: 1. Ethyl Ester Concentrates and 2. VDFO.

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1.2 FATTY ACID COMPOSITION

INTRODUCTION

Marine oil based Biomedical Test Materials (BTM) are characterized primarily by their fatty acid composition. Fatty acid composition is used by the BTM Program to select starting materials for production, to monitor production methods, to evaluate storage stability and assure composition of final products. A complete fatty acid profile is included with the QA report that accompanies each shipment of BTM to investigators.

Gas liquid chromatography (GLC) is probably the most widely used technique for the analysis of marine lipids. In 1986, when the BTM Program was initiated, existing official methods for fatty acid compositional analyses by GLC were methods developed primarily for vegetable oils and specified the use of packed columns (1). These methods were considered inadequate for the analysis of marine oil. The analytical approach adopted by the BTM Program was the use of capillary columns to achieve adequate separation for detailed composition and the use of an internal standard to permit quantitation of each component as an absolute amount of product. Consideration was also give to ease of analysis to permit routine determination of large numbers of samples.

Capillary column gas chromatography has been widely used in marine lipid research for approximately 20 years. Compared to packed columns, capillary columns provide many advantages for the analysis of marine fatty acids. Separation is significantly improved. The 60-80 fatty acids (C-12 to C-24 carbon chain lengths) commonly found in marine oils may be separated in 30-50 min. Background noise is reduced, relative to packed column chromatography, allowing detection of minor fatty acids. *Trans* fatty acids may be separated on capillary columns providing a quantitative measure of individual *trans* fatty acids, complimenting the analysis for total *trans* unsaturation by IR.

The increase in omega-3 research, made possible by the availability of test materials, and the establishment of labeling requirements for commercially available omega-3 nutritional supplements, emphasized the need for a collaboratively-tested, official method for the analysis of marine fatty acids. In 1988 an international collaborative study was initiated jointly by staff at the Charleston Laboratory and the Technical University of Nova Scotia. Based on the results of this study, the fatty acid analysis of marine oils using a capillary column with a bonded liquid phase has been adopted as an AOCS official method (Ce 1b-89) (2). It has also been recently adopted by the AOAC as an official method (3). The procedures specified in this official method are essentially those used by the BTM Program.

This method is suitable for all test materials produced by the BTM Program and can generally be applied to most fish oils. It should be noted that BF_3 catalyzed esterifications are not suited for samples containing polar plasmalogens, cyclopropane, cyclopropene, or furanoid fatty acids (4). If these types of fatty acids are expected, alkaline catalyzed esterification should be substituted .

PRINCIPLE

Fatty acid composition is determined by GLC, a technique based on partition of a solute between a mobile gas phase and a stationary liquid phase. For the purpose of GLC analysis, the fatty acid moieties of complex lipids are converted to simpler, more volatile fatty acid methyl esters (FAME). Prior to derivatization, an accurately weighed sample is mixed with a known amount of internal standard, providing a basis for quantitation in absolute amounts. Derivitazation is achieved by a two-step procedure. Triacylglycerols are transesterified with methanol by heating the sample with methanolic NaOH. The mixture is then reacted with boron trifluoride (in methanol) to convert any free fatty acids into FAME. The esters are extracted into isooctane for analysis by GLC, using a salting out technique (5). Ethyl ester concentrates and highly purified esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are analyzed as the ethyl esters without further derivitazation. Separation is achieved on a polar fused silica capillary column. Peak detection and measurement is by a flame ionization detector (FID). Peak areas are corrected for differences in detector response of the individual FAME using either theoretical correction factors (TCF) proposed by Ackman and Sipos (6) or empirical correction factors (ECF), as defined by Craske and Bannon (7). Comparison of relative retention times (RRT), with those of primary and secondary standards, chromatographed in the same manner as the samples, provides tentative identifications of the component fatty acids. Classical ancillary techniques such as equivalent chain length (ECL) value calculation, bromination, hydrogenation, argentation TLC, supplemented by instrumental analysis such as GC/MS are used for confirmation of identities (4). Amounts of individual fatty acids are calculated as a single-point ratio of the corrected peak area to that of the internal standard.

Each group of samples analyzed is accompanied by a reference material of similar composition to ensure accuracy of analysis. Regular participation in collaborative analyses, such as the Smalley Check Sample Program (AOCS, Champaign IL), provides continual evaluation of analyst and instrumentation.

APPARATUS

- Gas chromatograph (Hewlett Packard 5890 or equivalent), designed to accept capillary columns (0.22-0.32 mm ID), with an FID detector; computer controlled or with an electronic integrator. An autosampler is convenient, but optional

- Polar flexible fused silica capillary column (0.22-0.32 mm ID) at least 30 m in length, DB225, cyanopropylphenyl (J&W Scientific) or SUPELCOWAX-10, polyethylene glycol (Supelco)

- Centrifuge (Precision Centricone or equivalent) with rotor to accommodate 16 x 125 mm screw cap culture tubes and capable of at least 1300 rpm

- Nitrogen evaporator with water bath capable of maintaining 37°C (Meyers N-Evap or equivalent) situated in a fume hood

- Analytical balance
- Vortex mixer
- Pasteur pipettes, disposable
- Automatic positive displacement pipettor capable of delivering 1 ml ± 0.01 ml (Eppendorf repeater pipette No. 2226 000-6 or equivalent)
- Disposable culture tubes, 16 x 125 mm, with teflon lined-screw caps (Kimble #45066-A or equivalent)
- Temperature block modular heater, with block to accommodate 16 x 125 mm tubes and controlled temperature at 100 ± 2°C
- Hamilton syringe, 10 µl (specific for autosampler used or for manual injection)

REAGENTS

- Isooctane (ACS reagent grade)
- Methanol, absolute (ACS reagent grade)
- Boron trifluoride, 12% in methanol (Supelco)
- Sodium hydroxide (reagent grade), 0.5N in methanol
- Sodium chloride (reagent grade), saturated aqueous solution
- Butylated hydroxytoluene (BHT), reagent grade
- Methyl tricosanoate (23:0), 99+% (Nu Chek Prep, cat.#N-23-M)
- Ethyl tricosanoate(23:0), 99+% (Nu Chek Prep, cat. #N-23-E)
- GLC standards 68 and 87, 99+% (Nu Chek Prep)
- Ethyl esters standards - 18:0, 18:2n-6, 18:3n-3, 20:4n-6, and 22:6n-3, 99+% (Nu Chek Prep)
- Soft-gel encapsulated reference fish oil (Charleston Laboratory, BTM Program)
- Soft-gel encapsulated reference ethyl ester concentrate (Charleston Laboratory, BTM Program)
- Helium or hydrogen (99% pure or better), carrier gas

- Nitrogen, compressed, dried (for solvent removal and auxillary-up gas)

PREPARATION OF STANDARDS AND SAMPLES

I. Preparation of Standards:

A. Internal standard

Methyl esters - Transfer approximately 100 mg (weighed to the nearest 0.1 mg) of methyl tricosanoate into a 100 ml volumetric flask and bring to volume with isooctane containing 20µg/ml BHT.

Ethyl esters - Transfer approximately 400 mg (to the nearest 0.1 mg) of ethyl tricosanoate into a 100 ml volumetric flask and bring to volume with isooctane containing 20µg/ml BHT.

B. Primary standards

Methyl esters - Dissolve approximately 25 mg of GLC standards 68 and 87 in separate 1 ml volumes of isooctane.

Ethyl esters - Transfer accurately weighed amounts (to nearest 0.1 mg) of each standard into a volumetric flask and bring to volume with isooctane. Cost and availability of the standards must be considered in determining weights and volumes used. A mixed standard can be prepared by combining known volumes of each individual standard. Volumes of each standard should be adjusted to keep concentrations in line with those expected in the sample.

C. Reference materials

Weigh approximately 25 mg (to the nearest 0.1 mg) of oil or ester into a 16 x 125 mm screw-capped culture tube and prepare as specified below for samples.

II. Preparation of Samples:

A. Oils (fish and vegetable)

1. Weigh approximately 25 mg (to the nearest 0.1 mg) of oil into a 16 x 125 mm screw-capped culture tube.

2. Add 1.0 ml methyl tricosanoate internal standard (IS). Mix thoroughly and evaporate to dryness under a stream of N_2 using a nitrogen evaporator.

3. Add 1.5 ml of 0.5N NaOH, cap tightly, vortex, and heat for 5 min at 100°C in heating block.

4. Cool with tap water.

5. Add one ampule (2 ml) of boron trifluoride/methanol; cap tightly, vortex, and return the tube to the heating block for 5 min.

6. Cool to 37°C in water bath, add 1 ml isooctane and vortex.

7. Add 3 ml saturated NaCl and mix by repeatedly inverting the tube for approximately 1 min.

8. Centrifuge at 1300 rpm for approximately 2 min.

9. With a pasteur pipet, transfer the (upper) isooctane phase to a small vial containing anhydrous Na_2SO_4 (approximately a 2 mm layer).

10. Cap, shake, and allow to stand for approximately 20 min. Carefully transfer a portion of the isooctane containing the esters to an appropriate vial for GLC analysis, taking care to exclude the Na_2SO_4.

B. Ethyl esters (fish oil ester concentrates, highly purified esters of EPA and DHA and vegetable oil ethyl esters)

1. Weigh approximately 25 mg (to the nearest 0.1 mg) of oil into a 16 x 125 mm screw-capped culture tube.

2. Add 1.0 ml ethyl tricosanoate internal standard (IS), 1 ml of isooctane and mix thoroughly. Transfer to appropriate vial for GLC analysis.

DETERMINATION

I. Instrument Set-up

Injection System: The injection port should be fitted with an all-glass, split-injection liner. The split ratio used is approximately 1:80. A 1µl sample is injected manually using a 10 µl syringe (Hamilton) or by means of an autosampler, following the instructions for the particular model of instrument used. The injection port temperature is 250°C.

Carrier Gas: Hydrogen or helium, 99.999% pure. A moisture trap (Chrompak #7971 or equivalent) and an oxygen trap (Chrompak #7970 or equivatent) must be installed in the carrier line. An average linear velocity of 40 cm/sec for hydrogen or approximately 30 cm/sec for helium is used. Effects of temperature programming should be considered in setting this parameter.

Detector: The flame ionization detector should be designed for use with capillary columns. Hewlett-Packard instruments accommodate capillary columns by allowing for the addition of an auxillary gas (nitrogen) at the detector to compensate for the low flow rates of capillary columns. Some manufacturers do not have this requirement. If required, the combined flow

rate (carrier and auxillary) should be approximately 30 ml/min at the detector. Hydrogen and air flows to the detector should be set according to instructions for the model of instrument used. Detector temperature is 270°C.

Column oven: Temperature programming is recommended. Operating temperatures will be dependent on the liquid phase, length and age of the column. The GC used at the Charleston Laboratory has two capillary injection ports and two FIDs, permitting the installation of both a SUPELCOWAX-10 (30 m) and a DB225 (30 m) column in a single oven. This arrangement accommodates the analysis of BTM samples as well as a large number of samples generated through other projects/programs. Simultaneous separations for methyl esters, on DB225, and ethyl ester concentrates, on SUPELCOWAX-10, are achieved using the following program:

Initial temperature	170°C
Hold time	0
Rate	1°/min
Final temperature	225°C
Total time	55 min

It should be noted that for analysis of methyl esters on SUPELCOWAX-10 under these conditions, the 22C monoenes are not separated from geometric and positional isomers of 20:5n-3 that may be found in thermally abused oils, and 24C monoenes may coelute with 22:6n-3. For analysis of ethyl esters on DB225, under these conditions, 20:4n-3 and 20:5n-3 coelute as does 22:5n-3 and 22:6n-3.

II. Instrument performance evaluation/calibration:

1. Analysis of primary standards and reference materials

   Methyl esters -
   Analyze primary standards 68 and 87 and the soft-gel encapsulated reference fish oil.

   Ethyl esters -
   Analyze mixed standard prepared from primary standards and the soft-gel encapsulated reference ester concentrate.

2. Identification of FAME in standard chromatograms:

Tentatively identify the component esters from primary standards and reference material based on known elution order. Figures 1.2-1 and 1.2-2 illustrate typical separations. Retention times for the same component may vary somewhat from sample to sample, especially if amounts of the component are differ substantially. They are usually reproducible to within $\pm$ 2%. Relative retention times may be calculated using Equation I. Ancillary identification techniques should be applied as required.

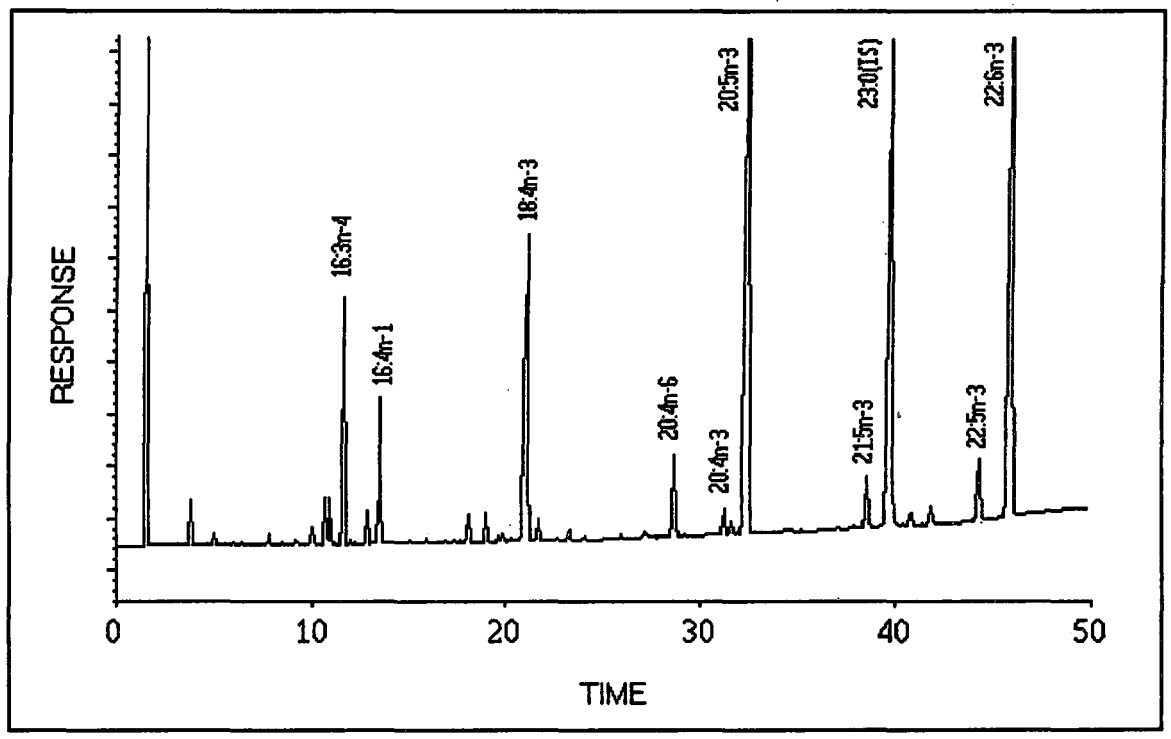

FIGURE 1.2-1. GLC Separation of Ethyl Ester Reference on SUPELCOWAX-10.

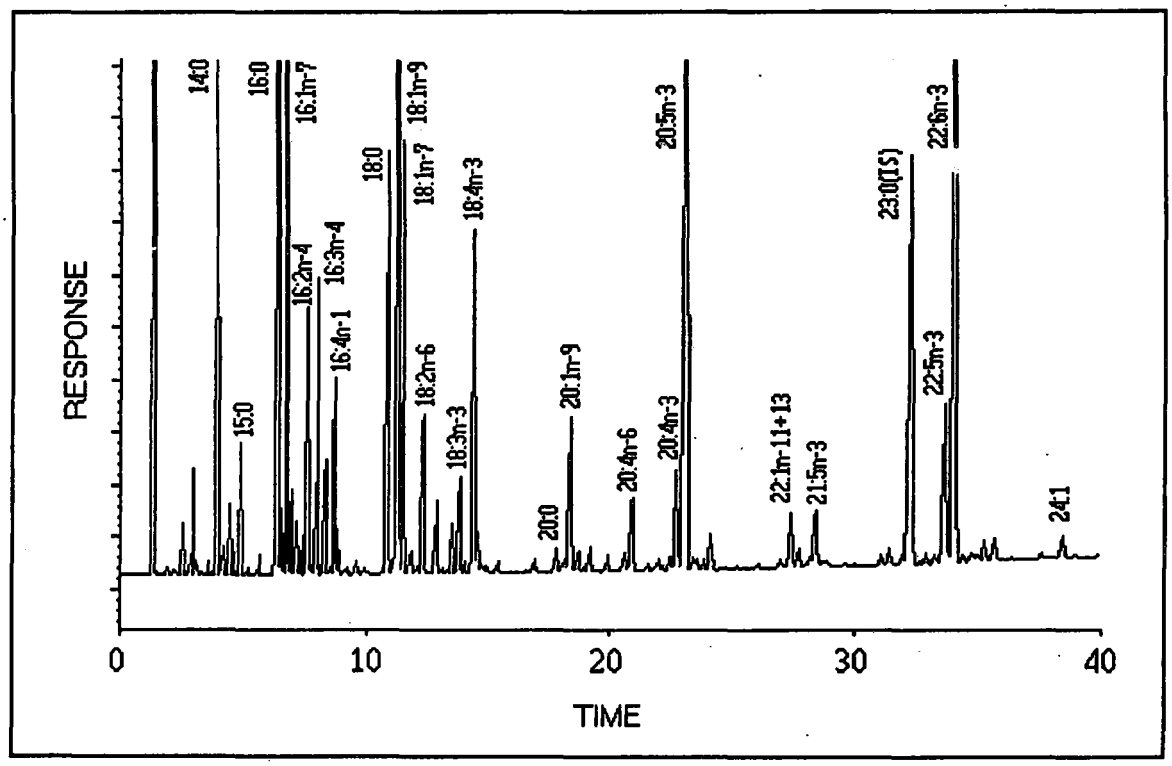

FIGURE 1.2-2. GLC Separation of TG Reference on DB-225.

3. Determination of correction factors:

Using Equation II, calculate correction factors for all component esters in the primary standards. Average the values for three runs each for the two standards. Ideally, the FID correction factors, calculated relative to 18:0, should conform to TCFs proposed by Ackman and Sipos (6) (Table 1.2-1). If deviations from theoretical values are observed, measures should be taken to achieve instrument optimization. Factors affecting deviation from theoretical detector response are discussed by Albertyn et al. (8), Craske and Bannon (9), and Bannon et al. (10).

Often it is not practical to completely optimize the instrument for theoretical response and ECFs can be used to account for slight deviations from theoretical values. Since plots of correction factors (theoretical and empirical) vs carbon number for saturates, monounsaturates, and polyunsaturates give fairly smooth curves, fatty acids for which standards are not available may be assigned the average ECF of the nearest preceeding and following known standards. This gives a reasonable approximation and is preferable to assigning a value of unity (11). Figure 1.2-3 illustrates the deviation of ECFs from TCFs for saturated FAME analyzed on DB225. Because of the difficulty in obtaining and maintaining pure standards of polyunsaturated fatty acid esters, the values obtained for ECF should be carefully scrutinized. Purity should be suspect for standards with ECFs that deviate from a smooth curve. Commercial standards should be evaluated for purity by GLC of the methyl esters and by quantitative TLC (Iatroscan TLC/FID) (12).

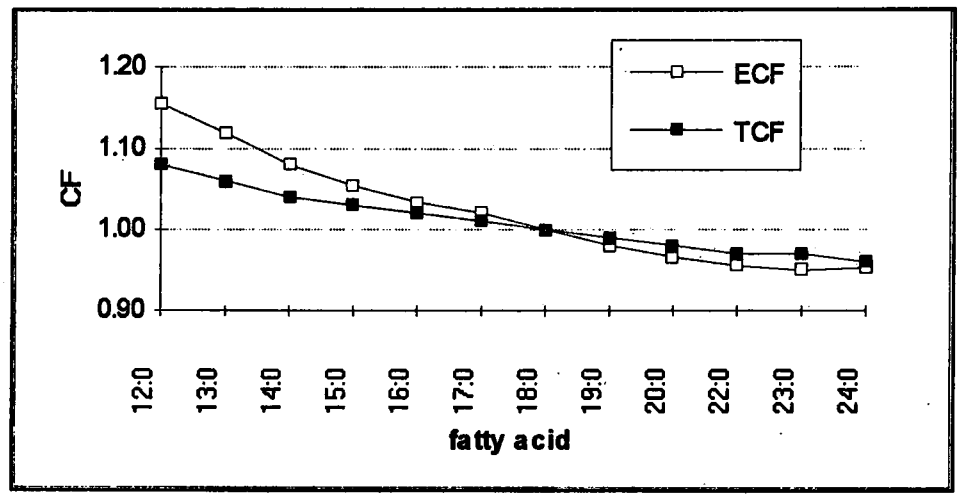

FIGURE 1.2-3. TCF/ECF vs Carbon Chain Length.

4. Preparation of calibration files

Most GC systems currently in use are capable of automated peak identification based on a retention time window, and quantitation by an internal standard method using either single-point or multi-point ratios. Using the retention time data obtained in step II and correction factors obtained in step III, set-up a calibration file following manufacturers guidelines for the specific instrument used.

III. Sample analysis:

1. Analyze samples by GLC using the same parameters as for analysis of standards. Each group of samples analyzed should be accompanied by a reference material. Identifications and composition computed automatically should be verified. In the absence of the capability for automated computation, RRT can be can be used for tentative identification (Equation I) and composition for individual ethyl esters or esterified fatty acids can be calculated using Equations III and IV.

CALCULATIONS

EQUATION I.

$$RRT_x = \frac{(RT_x - RT_s)}{(RT_{18:0} - RT_s)}$$

$RT_{18:0}$ = retention time of 18:0
RRT_x = relative retention time for component x
RT_x = retention time of component x
RT_s = retention time of solvent

EQUATION II.

$$K_{(x)} = \frac{A_{18:0} \times W_x}{W_{18:0} \times A_x}$$

K_x = correction factor for component x
$A_{18:0}$ = area of 18:0 in reference chromatogram
$W_{18:0}$ = weight of 18:0 in reference chromatogram
A_x = area of component x in reference chromatogram
W_x = weight of component x in reference chromatogram

EQUATION III.

$$FAE_x, mg/g = \frac{A_x \times K_x \times W_{IS} \times K_{IS}}{A_{IS} \times W_S}$$

FAE_x = fatty acid ester x
A_x = area of fatty acid x
K_x = correction factor for component x
W_{IS} = weight of IS in mg
K_{IS} = correction factor for IS
A_{IS} = area of IS
W_S = sample weight in g

EQUATION IV.

$$FA_x, mg/g = \frac{A_x \times K_x \times W_{IS} \times K_{IS} \times C_x}{A_{IS} \times W_S}$$

FA_x = fatty acid x
A_x = area of fatty acid x
K_x = correction factor for fatty acid x
W_{IS} = weight of IS in mg
K_{IS} = correction factor for IS
C_x = conversion factor necessary to express component as the fatty acid (See Table 1.)
A_{IS} = area of IS
W_S = sample weight in g

PRECISION

The relative standard deviation for duplicate analyses should be $< \pm 3\%$.

TABLE 1.2-1 Factors Used in Fatty Acid Calculations.

FATTY ACID	CF/18:0	MW (ME)	C (ME)*	MW(EE)	C (EE)*
10:0	1.10	186.3	0.93	200.3	0.93
12:0	1.08	214.3	0.93	228.3	0.94
12:1	1.07	212.3	0.93	226.3	0.94
13:0	1.06	228.4	0.94	242.4	0.94
14:0	1.04	242.4	0.94	256.4	0.95
14:1	1.04	240.4	0.94	254.4	0.94
15:0	1.03	256.4	0.95	270.4	0.95
15:1	1.02	254.4	0.94	268.4	0.95
16:0	1.02	270.5	0.95	284.5	0.95
16:1	1.01	268.4	0.95	282.4	0.95
16:2	1.00	266.4	0.95	280.4	0.95
16:3	1.00	264.4	0.95	278.4	0.95
16:4	0.99	262.4	0.95	276.4	0.95
17:0	1.01	284.5	0.95	298.5	0.95
17:1	1.00	282.5	0.95	296.5	0.95
18:0	1.00	298.5	0.95	312.5	0.96
18:1	0.99	296.5	0.95	310.5	0.95
18:2	0.99	294.5	0.95	308.5	0.95
18:3	0.99	292.5	0.95	306.5	0.95
18:4	0.97	290.4	0.95	304.4	0.95
19:0	0.99	312.5	0.96	326.5	0.96
19:1	0.99	310.5	0.95	324.5	0.96
20:0	0.98	326.6	0.96	340.6	0.96
20:1	0.98	324.5	0.96	338.5	0.96
20:2	0.97	322.5	0.96	336.5	0.96
20:3	0.97	320.5	0.96	334.5	0.96
20:4	0.96	318.5	0.96	332.5	0.96
20:5	0.95	316.5	0.96	330.5	0.96
21:0	0.98	340.6	0.96	354.6	0.96
21:5	0.95	330.5	0.96	344.5	0.96
22:0	0.97	354.6	0.96	368.6	0.96
22:1	0.97	352.6	0.96	366.6	0.96
22:2	0.96	350.6	0.96	364.6	0.96
22:3	0.96	348.6	0.96	362.6	0.96
22:4	0.95	346.5	0.96	360.5	0.96
22:5	0.94	344.5	0.96	358.5	0.96
22:6	0.94	342.5	0.96	356.5	0.96
23:0	0.97	368.6	0.96	382.6	0.96
24:0	0.96	382.7	0.96	396.7	0.96
24:1	0.96	380.6	0.96	394.6	0.96
24:5	0.94	372.6	0.96	386.6	0.96

* See Equation IV

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1.3 FREE FATTY ACIDS

INTRODUCTION

The AOCS method (1) for determination of free fatty acids (FFA) in oil samples is the recommended method of the International Association of Fish Meal Manufacturers (IAFMM) (2). It has often proven unsatisfactory for the analysis of marine oils which may be yellow or red due to carotenoids, or brown due to oxidation products. In addition, the endpoint color of the phenolphthalein indicator may be masked by slight turbidity in marine oils or may exhibit endpoint fading, leading to difficulty in completing the titration accurately. Despite special precautions, such as multiple determinations, great difficulty may be experienced in obtaining consistent and accurate values for the FFA content of marine oil samples. An improved titrimetric method for the determination of FFA in fish oils has been published (3,4) and is used by the BTM program.

PRINCIPLE

Free carboxylic acids are titrated to endpoint with 0.05N NaOH. Meta cresol purple (MCP) is used as the indicator and a ternary mixture of chloroform:methanol:isopropanol is used as the solvent system. Meta cresol purple turns from yellow to purple at endpoint, which avoids the problem of color interference from some marine oils. The mixed solvent system completely dissolves marine oil samples and mixes with at least 12 ml of aqueous NaOH titrant before becoming turbid. This method requires a much smaller sample size (1 g) than the AOCS method (up to 50 g) at low levels of free fatty acids. The titrant volume consumed by the titration is used to calculate the free fatty acid content as percent oleic acid.

APPARATUS

- 5 ml micro burette
- Analytical balance
- Beakers or Erlenmeyer flasks (disposable or glass)
- Disposable plastic transfer pipets
- 2 L volumetric flask
- 100 ml graduated cylinder
- Stir plate (magnetic)
- Stir bars (magnetic)

REAGENTS

- Solvent:
  Chloroform: methanol:isopropanol (ACS grade), 2:1:2

- Indicator:
  Meta cresol purple (MCP), 0.5% in distilled water (Fisher)

- Titrant:
  NaOH, 0.05N (Fisher)

- Standard:
  Oleic acid, 99.5% pure (Nu-Chek Prep)

DETERMINATION

1. Blank - Put 75 mls solvent mixture and 4 drops of MCP indicator in a beaker. Swirl. Place beaker on the stir plate and titrate with 0.05N NaOH to the MCP purple endpoint. The blank titration should be about 0.15 ml of 0.05N NaOH. If more than 0.2 ml of NaOH is required, adjust the pH of the MCP using dilute NaOH or HCl.

2. Sample - Weigh 1 g oil (to the nearest 0.005 g) in a 100 ml beaker. Add 75 ml of solvent mix and 4 drops of MCP indicator. Swirl. Titrate with 0.05N NaOH to endpoint.

An oleic acid standard is titrated, in duplicate, with each group of samples analyzed to assure accuracy of analysis.

CALCULATION

The percentage of FFA is calculated as oleic acid using the following formula:

$$\text{FFA, \% as oleic} = \frac{(S-B)\ x\ 1.41}{W}$$

where S = titration volume of Sample
B = titration volume of Blank
W = Weight of Sample (g)

PRECISION

The standard deviation obtained by the manual method of Ke *et al.* for concentrations of FFA up to 13% is <1.5% of the FFA value. This is greatly reduced over that of the AOCS method, i.e., 11% of the FFA value by different analysts and 6% of the average FFA value by the same analyst.

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1.4 ISOLATED TRANS FATTY ACIDS

INTRODUCTION

In most vegetable oils the naturally occurring unsaturated constituents contain only isolated, i.e., non-conjugated, double bonds in the *cis* configuration. Marine oils may naturally contain some *trans* isomers. During extraction and processing *cis* bonds may be isomerized to the *trans* configuration. Oxidation also promotes isomerization from the naturally occurring *cis* to the *trans* isomers (1). Production methods used by the BTM Program are specifically designed, and carefully controlled, to minimize lipid oxidation and the formation of geometric or positional isomers. Because of health concerns related to *trans* fatty acids in the diet, a level <5% of total *trans* unsaturation has been specified for all BTM products (2).

Both the AOCS (3) and the AOAC (4) have an official method for the determination of isolated *trans* isomers of fatty acids by infrared spectroscopy (IR); the AOAC uses the AOCS official method. The AOCS method is applicable to the determination of isolated *trans* bonds in natural or processed long-chain acids, esters, and triacylglycerols which contain only small amounts (< 5%) of conjugated materials and do not contain functional groups which modify the intensity of the C-H deformation about the *trans* double bond, such as castor oil containing ricinoleic acid or its geometrical isomer ricinelaidic acid (12-hydroxy-*trans*-9-octadecenoic acid). Firestone and LaBouliere (1) have demonstrated, by analysis of spiked samples, that spectra of triacylglycerols with 0 or low *trans* content produce isolated *trans* values that are 2-3% high while spectra of methyl esters produce values that are 1.5-3% low and suggest using an average of the two values. The AOAC method, used by the BTM Program to measure total *trans* unsaturation, specifies that long-chain fatty acids containing less than 15 % isolated *trans* isomers be converted to their methyl esters before analysis by IR.

PRINCIPLE

Isolated *trans* bonds in long-chain fatty acids, esters and triacylglycerols are measured by IR. An absorption band with maximum at about 10.3μ, arising from a C-H deformation about a *trans* double bond, is exhibited in the spectra of all compounds containing an isolated *trans* group. This band is not observed in the spectra of the corresponding *cis* and saturated compounds. Measurement of the intensity of this absorption band under controlled conditions is the basis of a quantitative method for the determination of isolated *trans* content.

A reference material accompanies each group of samples analyzed. The detection limit set for BTM Program analyses is < 5% total trans unstauration.

APPARATUS

- Fume Hood
- Infrared spectrophotometer, dual beam, covering the spectral region about 9 to 11μ, with wavelength scale readable to 0.01μ, and equipped with cell compartment holding 0.2 to 2.00 mm cells (Perkin Elmer 1420 or equivalent)
- Absorption cells, fixed thickness, 0.2 to 2.00 mm, NaCl or KBr windows. For use in null type instruments, pairs of cells matched to within 0.01 absorbance units are required. In split-beam type instruments, electronic balance of the two beams with both cells filled with CS_2 to within 0.01 absorbance units should be attained.
- Volumetric flasks, 5 ml and 10 ml volumes
- Centrifuge (Precision Centricone or equivalent) with head to accommodate 16 x 125 mm screw-capped vials and operating at a speed of at least 1300 rpm
- Hypodermic syringes with luer-lock fitting, for filling absorption cells
- Chart paper, calibrated in either transmittance or absorbance
- Analytical balance (Mettler AE200 or equivalent) capable of weighing ± 0.0002 g
- Nitrogen evaporator (Meyers N-Evap, or equivalent) with water bath capable of maintaining 37°C
- Disposable culture tubes, 16 x 125 mm, with teflon-lined screw caps (Kimble #45066A or equivalent)
- Temperature block modular heater, with block to support 16 x 125 mm tubes and controlled temperature at 100°C

REAGENTS

- Carbon disulfide, dry, ACS grade
- Primary standards: methyl elaidate, methyl oleate, ethyl elaidate , and ethyl oleate, >99% pure (Nu-Chek Prep)
- Secondary standards: the secondary standards are calibrated against the primary standard and are used in order to conserve the primary standard. Other acids, esters and triglycerides may be used as long as they contain a defined proportion of *trans*-isomer as determined by calibration with the primary standard.
- Sodium sulfate, anhydrous (reagent grade)

- Isooctane (ACS reagent grade)
- Methanol, absolute (ACS reagent grade)
- Boron trifluoride (Supelco), 12% in methanol
- Sodium hydroxide (reagent grade), 0.5N in methanol
- Nitrogen, compressed, with in-line moisture trap
- NaCl (reagent grade), saturated aqueous solution

PREPARATION OF STANDARDS & SAMPLES

I. Standards

1. Prepare standards containing 5, 10 and 15% methyl elaidate in methyl oleate for the determination of triacylglycerols and 5, 10, and 15% ethyl elaidate in ethyl oleate for determination of ethyl ester concentrates.
2. Weigh to the nearest 0.0001 g approximately 0.08g of standard into a 16 x 125 mm glass culture tube. Add 1 ml CS_2 and mix.

II. Triacylglycerols

1. Weigh approximately 0.100 g of oil (to the nearest 0.0001g) into 16 x 125 mm culture tube.
2. Add 4 ml 0.5N methanolic NaOH. Cap tightly, vortex and heat in heater block at 100°C for 7 min.
3. Cool tube with tap water.
4. Add one 5 ml ampule BF_3/methanol (12%), cap tightly, vortex and return to heater block for 5 min.
5. Cool tube to 37°C in water bath; add 2 ml isooctane and vortex.
6. Add 3 ml saturated NaCl and extract esters into isooctane by repeated inversion of the tube by hand for approximately 1 min. Vigorous mixing at this stage may form an emulsion.
7. Centrifuge at 1300 rpm approximately 2 min.
8. With a Pasteur pipet, transfer the upper, isooctane layer to another tube containing anhydrous Na_2SO_4 (approximately 2 mm layer).

9. Cap, shake, allow to stand at least 20 min.

10. Filter (Whatman No. 1) the clear isooctane solution into a dry tared tube. Place the tube in a 37°C water bath; evaporate the solvent under a stream of dry N_2. Determine weight of recovered esters.

11. Add 1 ml carbon disulfide (stored over molecular sieves), mix thoroughly.

III. Esters

1. Weigh 0.08 g (to the nearest 0.0001 g) ester concentrate into a 16 x 125 mm culture tube.

2. Add 1 ml carbon disulfide and mix. Add anhydrous sodium sulfate, mix, allow to stand 20 min, filter (Whatman No. 1) into clean, dry tube.

DETERMINATION

1. Fill matching absorption cells with CS_2 using a hypodermic syringe. With the cell in an upright position, inject the sample from the bottom allowing any trapped air bubbles to pass up through the cell. Cap securely to prevent evaporation of solvent. All operations involving transfer of CS_2 should be conducted in a fume hood.

2. Place the cells in the reference and sample beam holders of the spectrophotometer. Scan from 150.0 to 60.0μ to check baseline and cleanliness of the cells.

3. Remove the CS_2 from the sample cell

4. Flush cell with the standard solution, fill the cell with mixed standard and place in sample beam holder. With the reference cell containing CS_2 scan from 150.0 to 60.0μ. Once a curve has been obtained for the required standard, it need not be repeated as long as the same instrument can be used with the same programming controls. However, if for any reason the exact programming cannot be duplicated when measuring a specific sample, a new calibration curve must be obtained. A reference sample should be analyzed with each group of samples on a routine basis.

5. Flush the cell with CS_2,followed by the sample solution. Fill cell and place the cell in the sample beam holder and obtain duplicate scans.

6. If no measurable peak is observed at 10.3μ, the sample is reported as <5% *trans* unsaturation. If a measurable peak is observed, calculate the % *trans* unsaturation for each of the scans.

CALCULATION

1. On the chart, draw a baseline from 10.02μ to 10.59μ (Figure 2.3-1). Subtract the absorbance at the baseline (a) from the absorbance at 10.3μ maximum (b).

   Percent *trans* fatty acids is calculated by the following equation:

$$\%trans = \frac{a(sample)}{a(standard)} x100$$

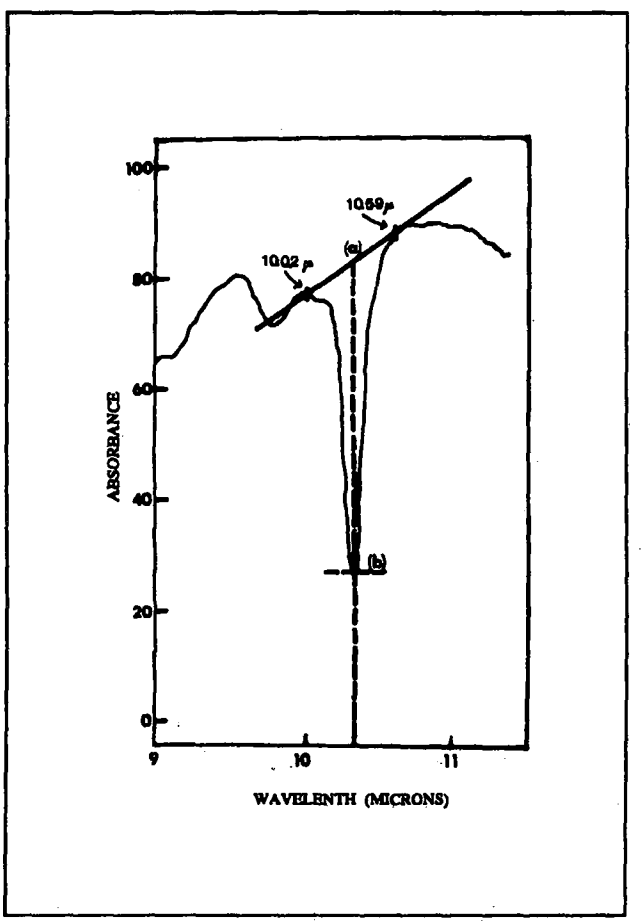

FIGURE 1.4-1. Infrared Scan of Methyl Elaidate.

Where,

a = absorptivity = A/bc
and,

A = absorbance
b = internal cell length in cm
c = concentration of solution, g/l

PRECISION

For duplicate scans the relative standard deviation is <5%.

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1.5 UNSATURATED FATTY ACIDS (IV)

INTRODUCTION

The iodine value is a measure of the unsaturation of fats and oils. The official method of both the AOCS and the IAFMM for the determination of iodine value is based on the Wijs Method (1,2). The BTM Program uses this method, modified for autotitration to a voltametric endpoint (3). An additional modification, is the use of chloroform as the solvent rather than carbon tetrachloride as specified in the AOCS method.

PRINCIPLE

An oil sample is incubated for 1 hr in the dark with an excess of iodine in acetic acid (Wijs solution). The amount of iodine absorbed is proportional to the number of double bonds present in the oil and is determined by titrating the excess iodine with sodium thiosulfate. The iodine value is expressed as grams of iodine absorbed per 100 grams of sample. The method is applicable to all normal fats and oils not containing conjugated systems.

APPARATUS

- Autotitrator (Mettler DL-20 or equivalent)
- Mettler DM140 electrode, or equivalent capable of determining ± 0.05 pH units
- 20 ml burette (Mettler)
- Printer (Epson FX-85 or equivalent)
- 250 ml glass titration flasks (Mettler ME-23515 or equivalent) with caps
- Analytical balance, capable of weighing to $\pm$ 0.0001g, with RS232 interface (Mettler AE200 or equivalent)
- Automatic pipet (Rainin Pipetman or equivalent)
- 100 ml disposable titration beakers
- Graduated cylinders, 100 and 500 ml

REAGENTS

- Potassium iodide (KI) (ACS grade), 150 g/L in distilled H_2O (or 37.5 g KI in 250 ml distilled H_2O). The H_2O should be de-gassed by boiling and then cool to room temperature before making the solution. This solution should be prepared weekly.

- Potassium iodide (KI) (ACS grade), saturated. 30 g/21 ml boiled degassed, distilled H_2O. This solution, used for the determination of titrant concentration, has a shelf-life of one week (Note 1).

- Starch indicator solution: 1.0% soluble starch in distilled H_20 (Fisher).

- Glacial acetic acid:Chloroform, 3:2 (V:V) (reagent grade)

- Chloroform (ACS grade)

- Wijs iodine solution (Fisher). If the solution turns dark brown as it is added, discard, and use a fresh bottle.

- Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$), ACS grade, 0.1N in distilled H_2O (50 ml of the 1N stock solution is diluted to 500 ml with distilled H_20).

- Potassium iodate (KIO_3) 1 N standard solution (Fisher)

- Potassium iodate (KIO_3) 0.1 N solution

DETERMINATION

1. Prepare fish oil and ester concentrate samples for analysis by assuring that no particulate matter or moisture is present; filtration may be performed.

2. The weight of the sample must be such that there will be an excess of Wijs solution of 50-60% of the amount added, i.e. 100-150% of the amount absorbed. TABLE 1.5-1 provides a guide to the sample weight required for determination at different iodine values. Although the range of iodine values given in the table does not include the values that may be expected for ethyl ester concentrates and highly purified EPA and DHA, the required weights for these products can be extrapolated.

TABLE 1.5-1. Sample Weights Required For Iodine Value Determination (AOCS, 1989).

EXPECTED IODINE VALUE	SAMPLE WEIGHT (g)		WEIGHTING ACCURACY (± g)
	100% EXCESS	150% EXCESS	
<3	10.000	10.000	0.001
3	10.576	8.4613	0.005
5	6.3460	5.0770	0.0005
10	3.1730	2.5384	0.0002
20	1.5865	0.8461	0.0002
40	0.7935	0.6346	0.0002
60	0.5288	0.4231	0.0002
80	0.3966	0.3173	0.0001
100	0.3173	0.2538	0.0001
120	0.2644	0.2115	0.0001
140	0.2266	0.1813	0.0001
160	0.1983	0.1587	0.0001
180	0.1762	0.1410	0.0001
200	0.1586	0.1269	0.0001

3. Calibrate the electrode using standard buffer solutions.

4. Titrant concentration - determine concentration of the titrant daily. Set autotitrator with the configuration show in Table 1.5-2. Enter 12.69 for "const", 2.0 for "const reag", and 0 for "blank." Switch the instrument to concentration mode. Enter a value of "1" at the prompt for weight. Pipet 5 ml 0.1N KIO_3 into a disposable titration beaker. Add 30 ml acetic acid:chloroform solution. Add 0.5 ml saturated KI solution and start a timer. Place the beaker on the titrator and press "run." At the end of exactly one minute, add 30 ml distilled H_2O and 5 ml starch indicator solution. The titrant concentration should be between 1.9-2.1 meq/burette vol. (=0.1 N x 20 ml burette vol.)

5. Blank - pipet 20 ml chloroform into a 250 ml titration flask. Swirl and cap. Always run at least 1 blank with each group of samples.

6. Samples - Using TABLE 1.5-1 as a guide, accurately weigh an appropriate amount of oil or ester into a 250 ml titration flask. Add 20 ml of chloroform. Swirl and cap. Caps may need to be vented after swirling, due to pressure buildup.

TABLE 1.5-2. Autotitrator Configuration for Determination of Titrant Concentration.

Parameter No.	Value	Description
1	2	Titration mode: equivalence point
2	1	Reaction type: standard titration
3	0	No predispensing
4	0.3	Maximum volume in burette volumes
5	0	mV endpoint
6	75	Stirring time
7	11	Report: titration curve, initial signal
8	1	Output to printer

7. Pipet 25 ml (using a volumetric pipet) of the Wijs solution into all blank and sample titration flasks at 10 minute intervals. Be sure the pipet tip does not touch the solution as the Wijs is being added. Swirl and re-cap. Caps should be vented again.

8. Immediately store all flasks in a dark place, each for exactly 1 hour at 25 (±5)°C.

9. Remove the flasks from storage and add 20 ml of KI solution, followed by 100 ml of distilled H_2O. This stops the chemical reaction.

10. Configure the DL-20 autotitrator with the settings listed in Table 1.5-3. The values for "const" and "const reag" (12.69 and 2.0 respectively), are retained in memory.

TABLE 1.5-3. Autotitrator Configuration for Sample Analysis

Parameter No.	Value	Description
1	2	Titration mode: equivalence point
2	1	Reaction type: standard titration
3	0	No predispensing
4	2(sample); 3(blank)	Maximum volume in burette volumes
5	0	mV endpoint
6	15	Stirring time
7	11	Report: titration curve, initial signal
8	1	Output to printer

11. Switch the instrument to blank mode. Place the titration flask on the instrument and run the blank(s). The value should be 4.8-5.4. If it is <4 or >6, all samples must be discarded, and blanks and samples reprepared using a fresh bottle of Wijs solution. This blank value is automatically stored in the autotitrator's memory and used for all subsequent calculations.

12. Place the sample titration flask on the instrument. Manually enter the sample weight, and run each samples.

CALCULATION

In the AOCS standard method, the iodine value is calculated as follows:

$$IV = (B-S) \times N \times \frac{12.69}{W}$$

where,

B =	volume of titrant used for blank determination
S =	volume of titrant used for sample determination
N =	normality of titrant
12.69 =	meq weight of iodine x 100
W =	weight of sample, in g

The autotitrator reports the iodine value in % iodine absorbed.

PRECISION

For samples analyzed in triplicate, the relative standard deviation is routinely less than 2%.

NOTES

1. Store saturated KI solution in the dark. Determine the concentration of the KI solution daily by adding 2 drops of starch indicator solution to 0.5 ml of KI solution in 30 ml acetic acid:chloroform. This solution should turn blue. If this requires more than 1 drop of 0.1 N sodium thiosulfate to discharge, discard the KI solution and prepare a fresh bottle. If the KI solution turns yellow or a lot of precipitate forms in it, discard.

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1.6 DEUTERATED FATTY ACIDS

INTRODUCTION

Long chain highly unsaturated vinyl deuterated fatty acids have been virtually unavailable to investigators interested in n-3 fatty acid research. Because of their lack of availability, standard methods have not evolved for the QA of these compounds. Therefore a combination of analytical methods applicable to fatty acids along with standard chemical investigation for structure and molecular integrity are applied to the QA of such vinyl deuterated fatty acids as linolenic acid (18:3n-3, d6), arachidonic acid (20:4n-6, d8), eicosapentaenoic acid (20:5n-3, d10), docosahexaenoic acid (22:6n-3, d12).

PRINCIPLE

The quality assurance of vinyl deuterated n-3 fatty acids is accomplished using a variety of analytical methodologies. The product is tested by TLC for presence of free fatty acids, fatty acid esters and other components. If TLC indicates that the product is relatively pure, then the sample is analyzed for fatty acid composition utilizing capillary GC. If the elution pattern is consistent with the presence of a single component (>98%) and the elution time is identical with an authentic standard for the compound the product is then subjected to GC/MS, NMR and IR: the mass is determined (GC/MS), the deuterated structure is verified (NMR) and the percent *trans* product (IR) is determined (<5%).

DETERMINATION

1. Conduct semi-quantitative TLC (1) to determine the number of major components present; if only one component is present as expected, verify that it is a free fatty acid.

2. Conduct TLC/FID to determine the amount of free fatty acid in the material (2).

3. Conduct preparative TLC to isolate the free fatty acid band for GC studies (3).

4. Prepare the fatty acid methyl ester (FAME) derivatives of the isolated free fatty acid band and analyze by GC (4). Identify and quantify all major and minor fatty acids present. Examine the data for shifts in relative retention time, for the specific FAME under study, from that of the non-deuterated fatty acid as an indication of deuteration.

5. Analyze the FAME by GC/MS (5) to verify the molecular weight as being compatible with that of the FAME if it were fully vinyl-deuterated. Compare the spectrum for the FAME under study with that for an authentic standard of the non-deuterated FAME to verify the fragmentation pattern. If a molecular ion is present, determine the molecular weight. Verify that <0.001% d_0 is present.

6. Analyze the FAME by NMR to verify the structure and the distribution of deuterium. Compare the NMR splitting pattern and chemical shifts with that of an authentic standard of non-deuterated FAME.

REFERENCES

1. Refer to Section 1.1 of this manual.

2. Ackman, R.G. (1981) Flame ionization detection applied to thin layer chromatography on coated quartz rods. Meth. Enzymol. 72:205-252.

3. Kates, M. (1975) Techniques of lipidology. Pp. 428-446. *In*, Laboratory Techniques in Biochemistry and Molecular Biology, T.S. Work and E. Work (eds.), American Elsevier Publishing Co., New York, 610p.

4. Refer to Section 1.2 of this manual.

5. Support is provided by NIH/ADAMHA by providing verification of GC/MS studies.

2.0 CHOLESTEROL

INTRODUCTION

Marine oils contain significant amounts of cholesterol that are removed or greatly reduced during processing of the BTM oils and esters. Cholesterol content is measured at various stages during production of the test materials to monitor processing techniques, and in the final products to assure quality.

An official method for the gas chromatographic determination of cholesterol has received final action by the AOAC (1). This method requires isolation of the sterols by TLC prior to analysis by GC using a packed column. A simplified method for determination of cholesterol and some plant sterols in fishery-based products is described by Kovacs *et al.* (2). The method used by the BTM Program is a modification of a method developed at the USDA Human Nutrition Research Center in Beltsville, MD (3) that permits the determination of cholesterol, major plant sterols and α- and γ-tocopherols from a single analysis by capillary gas chromatography.

PRINCIPLE

A sample containing approximately 0.1 g of lipid (fat, oil or lipid extract) is mixed with a known weight of the internal standard (5-α-cholestane) and saponified at 80°C with aqueous KOH in the presence of pyrogallol. The non-saponifiables are extracted with cyclohexane, the solvent is removed under nitrogen, and the trimethylsilyl ethers are prepared using N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane in pyridine. This derivatized total non-saponifiable fraction is chromatographed on a non-polar fused silica wall-coated open-tubular (WCOT) column. Cholesterol is identified by comparison of its peak retention time relative to that of an internal standard, with those of cholesterol standards prepared and chromatographed in an manner identical to the samples. For routine analyses, a single point ratio is used to calculate the amount of cholesterol.

An analysis of a reference material accompanies each group of samples analyzed to ensure accuracy. Periodically, a spiked coconut oil (Standard Reference Material 1563, NIST, Gaithersburg, MD) is analyzed. Regular participation in collaborative analyses, such as the Smalley Check Sample Program (AOCS, Champaign, IL) provides continual evaluation of analyst and instrumentation.

APPARATUS

- Fume hood
- Gas chromatograph (HP5890 or equivalent), equipped for use with capillary columns (0.22-0.32 mm id), with an FID detector and optional autosampler
- Non-polar flexible fused-silica capillary column, Ultra 2, 25 m x 0.2 mm x 0.3μ film thickness (Hewlett-Packard), or DB-1, 60 m x 0.22 mm x 0.25μ film thickness (J&W Scientific)
- Centrifuge (Centra 4 International Centrifuge or equivalent) with rotor to accommodate 25 x 150 mm screw capped tubes, capable of operating at 1300 rpm
- Nitrogen evaporator with water bath capable of 37°C (Meyers N-Evap or equivalent)
- Water bath, 80°C (Precision Scientific or equivalent)
- Vortex mixer
- Pasteur pipettes, disposable.
- Automatic positive displacement pipet capable of delivering 1 ml $\pm$ 0.01 ml (Eppendorf Repeater Pipet Model No. 2226 000-6 or equivalent)
- Centrifuge tubes, 25 x 125 mm, with teflon lined screw caps
- Silanized 16 x 125 mm glass tubes with teflon-lined screw caps,
- Analytical balance (Mettler AE163 or equivalent)
- Mechanical sample mixer (Fisher-Kendall or equivalent)
- Hamilton syringe, 10μl (#1750 or equivalent)
- Moisture trap (Chrompak #7971 or equivalent)
- Oxygen trap (Chrompak #7970 or equivalent)
- Volumetric flask, 100 ml
- Glass transfer pipettes, 10 ml
- Solvent/reagent bottles fitted with calibrated dispensors
- Autosampler vials with conical inserts

REAGENTS

- Hexane, ACS reagent grade
- Isooctane, ACS reagent grade
- Butylated hydroxytoluene (BHT), reagent grade
- Nitrogen, compressed
- Potassium hydroxide solution - dissolve 30 g KOH (85%) in 20 ml distilled water.)
- Pyridine, silation grade (Pierce Chemical Co.)
- N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (Supelco Sylon BFT or equivalent). Sylon BFT is corrosive and will attack syringe needles and plungers, thus they should be rinsed promptly. Pyridine will cause some elastomers to swell (e.g., vitons). Glass and teflon are resistant to pyridine.
- Sylon CT (Supelco)
- Cholesterol standard (Eastman Kodak)
- 5-α-Cholestane, 99+% (Supelco)
- Absolute ethanol, reagent grade, degassed under vacuum using a rotary evaporator
- Pyrogallol, reagent grade (Malinckrodt), 3% w/v in degassed (under vacuum using a rotary evaporator) absolute ethanol
- Helium (99.999% pure or better), carrier gas
- Hydrogen (99.99% pure or better), FID
- Compressed air (breathing quality or better), FID
- Nitrogen, compressed, make-up gas
- Cyclohexane, degassed under vacuum using a rotary evaporator
- Encapsulated fish oil reference sample (Chas. Laboratory BTMP)

PREPARATION OF STANDARDS

1. **Internal Standard**: Accurately weigh approximately 50 mg of 5-α-cholestane and 20 mg of BHT, transfer to a 100 ml volumetric flask and bring to volume with isooctane.

Transfer 10 ml of this stock solution to a 100 ml volumetric flask and dilute to volume to give a working standard with a concentration of 40-50µg/ml 5-α-cholestane + 20µg/ml BHT.

2. **Primary Standard**: Accurately weigh approximately 50 mg of cholesterol, transfer to a 100 ml volumetric flask and make to volume with isooctane containing 1% pyridine (v/v). Transfer 10 ml of this stock solution to a 100 ml volumetric flask and dilute to volume to give a working standard with a concentration of 40-50µg/ml. Pipet 2.0 ml of the working standard and 2.0 ml of the internal standard into a centrifuge tube and proceed as outlined in preparation of samples, beginning with step 3. The volume of cholesterol should be adjusted, as required, to keep the concentration in line with that expected in the samples.

3. **Reference Sample:** Prepare as specified for samples, starting with Step 1.

PREPARATION OF SAMPLES

1. Weigh 80 - 100 mg (to the nearest 0.0001 g) of oil or ester concentrate into a 50 ml screw cap centrifuge tube.

2. Add 1.0 ml internal standard to each sample.

3. Evaporate the solvent under a stream of nitrogen using an N-Evap with a 37°C water bath.

4. Add 8 ml pyrogallol-ethanol solution and 0.5 ml saturated KOH.

5. Cap tubes tightly and vortex 5 sec.

6. Heat tubes for 8 min in 80°C water bath; shake vigorously after 1,2 and 4 min so that the solution washes tube sides and caps.

7. Remove tubes and cool ,under runing tap water, for 15 sec.

8. Add 10 ml cyclohexane and vortex for 5 sec.

9. Add 6 ml distilled water and shake for 15 min (mechanical mixer).

10. Centrifuge for 15 min (1300 x g).

11. Transfer cyclohexane layer to a 16 x 125 mm silanized culture tube taking care to exclude the lower phase.

12. Evaporate the solvent with a gentle stream of nitrogen using an N-Evap with a 37°C water bath.

13. Add 100µl pyridine and 100µl Sylon BFT; vortex 5 sec and let stand 15 min at room temperature. Transfer the sample to an autosample vial fitted with a 200µl conical insert for GC analysis.

DETERMINATION

The standards and samples are chromatographed isothermally on a fused silica capillary column (DB-1 or HP Ultra 2) using an all-glass split injection port with a split ratio of approximately 1:40. Carrier gas is helium (99.99% pure) with in-line moisture and oxygen traps. The average linear velocity is approximately 30 cm/sec. In instruments requiring make-up gas when using capillary columns, nitrogen is used as the auxillary gas to the flame ionization detector (FID). The combined flow rate at the detector (carrier + auxillary) is 30 ml/min. The following temperature parameters are used on the HP5890 GC: injection port - 300°C; Detector - 300°C; column oven - 280°C. A 1µl sample is injected either manually using a 10µl syringe or by autosampler.

CALCULATION

Most GC systems currently in use are capable of automated peak identification based on a retention time window, and quantitation by an internal standard method using either single-point or multiple-point ratios. Follow manufacturers guidelines for set-up of these procedures. Procedures for manual calculations follow:

The relative retention time is calculated for the cholesterol standard versus that of the internal standard (Equation I). The cholesterol peak is then identified in the sample by comparison of the relative retention time with that of the cholesterol standard.

A single-point ratio is used for calculation of cholesterol for routine analyses. (Equations II and III). However, linearity should be confirmed for the range of 0.5 - 1.5 times the amount expected in the samples.

EQUATION I.

$$RRT_{Ch} = \frac{RT_{Ch} - RT_S}{RT_C - RT_S}$$

RRT_{Ch} = relative retention time for cholesterol
RT_C = retention time of cholestane
RT_{Ch} = retention time of cholesterol
RT_C = retention time of solvent

EQUATION II.

$$K_{ch} = \frac{A_C \times W_{Ch}}{W_C \times A_{Ch}}$$

K_{Ch}	= correction factor for cholesterol
A_C	= area of cholestane in reference chromatogram
W_C	= weight of cholestane in reference chromatogram
A_{Ch}	= area of cholesterol in reference chromatogram
W_{Ch}	= weight of cholesterol in reference chromatogram

EQUATION III

$$Ch_{mg/g} = \frac{A_{Ch} \times K_{Ch} \times W_C}{A_C \times W_S}$$

$Ch_{mg/g}$	= amount of cholesterol
A_{Ch}	= area of cholesterol in the sample chromatogram
K_{Ch}	= correction factor for cholesterol
W_C	= weight of cholestane (μg) in sample chromatogram
A_C	= area of cholestane in sample chromatogram
W_S	= sample weight (mg)

PRECISION

The relative standard deviation for this assay is ± 5%.

REFERENCES

1. AOAC. (1984) Fats (animal) in vegetable fats and oils (determination of cholesterol), gas chromatographic method. (#28.110-28.117, Final action), Pp. 522-523. *In* Official Methods of Analysis of AOAC, 14th edition, S. Williams (ed.) Assoc. Ofc. Anal. Chem. Arlington, VA, 1141 p.

2. Kovacs, M.I.P, W.E. Anderson, and R.G. Ackman. (1979) A simple method for the determination of cholesterol and some plant sterols in fishery-based products. J. Fd. Sci. 44:1299-1305.

3. Slover, H.T., R.H. Thompson, Jr, and G.V. Merola. (1983) Determination of tocopherols and sterols by capillary gas chromatography. J. Am. Oil Chem. Soc. 60:1524-1528.

3.0 FATTY ACID OXIDATION PRODUCTS

3.1 PEROXIDES (PV) - Regular Method

INTRODUCTION

Peroxide value is used as a primary measurement of oxidation in oils. An official method exists in both the AOCS (1) and the AOAC (2) for the determination of the peroxide value. The AOCS method is used with an autotitrator by the BTM Program.

PRINCIPLE

This titrimetric method determines all substances which oxidize potassium iodide, in terms of milliequivalents of peroxide per 1000 grams of sample. These substances are assumed to be peroxides or other similar products of fat oxidation. The method is applicable to all normal fats and oils including margarine and is highly empirical. Any variation in procedure may result in variation in results.

APPARATUS

- Autotitrator, Mettler DL20 or equivalent
- Analytical balance, capable of measuring ±0.001 g, with RS232 interface
- Printer, Epson FX-85 or equivalent
- Electrode (Mettler DM140)
- 5 ml burette (Mettler)
- 100 ml disposable titration beakers
- Pipetman P1000 pipet and tips (or equivalent)
- Graduated cylinder, 50 ml
- Timer, 60 minute

REAGENTS

- Glacial acetic acid:chloroform, 3:2 (V:V) (reagent grade)
- Sodium thiosulfate ($Na_2S_2O_35H_20$), 0.05N (25 ml 1 N, QS to 500 ml with distilled H_20)
- Potassium iodide (KI) (saturated) (Fisher ACS grade), 30 g/21 ml boiled (degassed) distilled H_2O (Note 1) Lasts one week.
- Starch indicator solution: 1.0% soluble starch in distilled water. (Fisher)
- Potassium iodate (KIO_3), 0.1 N standard solution (Fisher)

DETERMINATION

1. Calibrate the electrode using standard buffer solutions.
2. Configure the autotitrator with the settings given below:

Parameter	Value	Description
1	2	Titration mode: equivalence point
2	1	Reaction type: standard titration
3	0	No predispensing
4	0.3	Maximum volume in burette volumes
5	0	mV endpoint
6	75	Stirring time
7	11	Report: titration curve, initial signal
8	1	Output to printer

3. Titrant concentration - determine concentration of the titrant daily. Enter 1000 for "const", 20 for "const reag", and 0 for "blank." Switch the instrument to concentration mode. At the prompt for weight, enter a value of "1". Pipet 0.5 ml KIO_3 into a disposable titration beaker. Add 30 ml acetic acid-chloroform solution. Add 0.5 ml saturated KI solution and start a timer. Place the beaker on the titrator and press "run." At the end of exactly one min, add 30 ml distilled H_20 and 5 ml starch indicator solution. The titrant concentration should be between 0.23 - 0.27 meq/burette vol. (= 0.05 N x 5 ml burette vol.).

4. Samples - tare a disposable beaker on the balance. Weigh approximately 5 g (± 0.05 g) oil into titration beaker. Press "run" to enter the weight on the autotitrator. Add 30 ml acetic acid-chlorform solution. Add 0.5 ml saturated KI solution and start a timer. Place the beaker on the titrator and press "run." At the end of exactly one minute, add 30 ml distilled H_20 and 5 ml starch indicator solution.

CALCULATION

The autotitrator automatically calculates the peroxide value as milliequivalents of peroxide per 1000 g of sample, using the following formula:

$$PV = \frac{(S-B) \times N \times 1000}{sample\ wt.}$$

where,

B = titration volume of blank
S = titration volumeof sample
N= normality of sodium thiosulfate solution

PRECISION

For samples analyzed in triplicate, the relative standard deviation is generally < 5%.

NOTES

1. Store saturated KI solution in the dark. Determine the concentration of the KI solution daily by adding 2 drops of starch solution to 0.5 ml of KI solution in 30 ml acetic acid:chloroform (3:2). If a blue color is formed which requires more than 1 drop of 0.1 N sodium thiosulfate solution to discharge, discard the iodide solution and prepare a fresh solution. If the solution turns yellow, discard and prepare fresh.

REFERENCES

1. AOCS (1989) Peroxide value (Cd 8-53, reapproved 1989). *In* Official and Recommended Practices of the American Oil Chemists Society, fourth edition, D Firestone (ed.). Am. Oil Chem. Soc., Champaign IL, 2p.

2. AOAC (1990) Peroxide value of oils and fats, titration method (965.33, Final action). P. 956. *In* Official Methods of Analysis of AOAC, vol. 2. 15th edition, K. Helrich (ed.). Assoc. Ofc. Anal. Chem., Arlington, VA, 1298 p.

3.2 PEROXIDES (PV) - Micro Method

INTRODUCTION

When peroxide values must be determined for limited amounts of sample (e.g., purified ethyl esters of EPA & DHA), it is not possible to use the official AOCS method (1). Therefore, a modified method for the micro-determination of lipid hydroperoxides (2) is used by the BTM Program.

PRINCIPLE

As in the AOCS method (1), the micro-titrimetric method determines all substances which oxidize potassium iodide, in terms of milliequivalents of peroxide per 1000 grams of sample. These substances are assumed to be peroxides or other similar products of fat oxidation. The method is applicable to all normal fats and oils including margarine and is highly empirical. Any variation in procedure may result in variation in results. Samples are continually blanketed with nitrogen and reagents are chilled to minimize lipid oxidation.

APPARATUS

- Autotitrator, Mettler DL20 or equivalent.
- Analytical balance, capable of measuring ±0.001 g, with RS232 interface.
- Printer, Epson FX-85 or equivalent.
- Electrode (Mettler DM140).
- 20 ml burette (Mettler).
- 250 ml glass titration flasks (Mettler ME-23515 or equivalent).
- Automatic pipet (Rainin Pipetman or equivalent) and tips.
- Graduated cylinder, 100 ml.
- Timer, 60 minute.

REAGENTS

- Glacial acetic acid: chloroform, 3:2 (V:V) reagent grade.

- Sodium thiosulfate ($Na_2S_2O_3 \cdot 5\ H_2O$), 0.001 N (1 ml 1 N, QS to 1000 ml with freshly distilled H_2O, nitrogen purged and chilled). Keep the titrant in an ice bath once it has been made up).
- Potassium iodide (KI), saturated (Fisher ACS grade), 30 g/21 ml boiled (degassed) distilled H_2O (Note 1) Lasts one week.
- Starch indicator solution: 1.0% of soluble starch in distilled H_2O (Fisher).
- Potassium iodate (KIO_3), 0.1 N standard solution (Fisher).
- Chilled, nitrogen purged, distilled H_2O.
- Ice
- Nitrogen line (low flow)

DETERMINATION

1. Calibrate the electrode using standard buffer solutions.
2. Configure the autotitrator with the settings given below.

Parameter	Value	Description
1	2	Titration mode: equivalence point
2	1	Reaction type: standard titration
3	0	No predispensing
4	3	Maximum volume in burette volumes
5	0	mV endpoint
6	300	Stirring time
7	11	Report: titration curve, initial signal
8	1	Output to printer

3. Insert nitrogen line through the hole provided for this purpose in the top cap of the titration head. This keeps samples continually blanketed with nitrogen, to minimize oxidation.
4. Titrant concentration - determine concentration of the titrant daily. Enter 1000 for "const," 20 for "const reag," and 0 for "blank." Switch the instrument to

concentration mode. At the prompt for weight, enter a value of 1. Pipet 0.5 ml KIO_3 into a glass titration flask. Add 30 ml acetic acid-chloroform solution. Add 0.5 ml saturated KI solution and start a timer. Manually swirl the flask for 30 sec, then place on the autotitrator. At the end of exactly 1 min, add 100 ml chilled, nitrogen purged, distilled H_2O and 5 ml starch solution. Press "run." Place an ice bath under the flask. When mV numbers begin appearing on the autotitrator (≈ 5 min) remove the ice bath. The titrant concentration should be between 0.0191 - 0.025 meq/burette vol. (= 0.001 N x 20 ml burette vol.)

5. Samples - change parameter 4 in the autotitrator to a value of 1. Tare a glass titration flask on the balance. Weigh approximately 0.5 g (±0.01 g) esters into titration flask. Press "run" to enter the weight on the titrator. Add 30 ml acetic acid: chloroform solution. Pipet in 0.5 ml saturated KI solution and start a timer. Place on the autotitrator. At the end of exactly 1 min, add 100 ml chilled, nitrogen purged, distilled H_2O and 5 ml starch indicator solution. Press "run." Place an ice bath under the flask. When mV numbers begin appearing on the autotitrator (≈ 5 min), remove the ice bath.

CALCULATION

The autotitrator automatically calculates the peroxide value as milliequivalents of peroxide value as milliequivalent of peroxide per 1000 g of sample, using the following formula:

$$PV = \frac{(S-B) \times N \times 1000}{sample\ wt.}$$

where, B = titration volume of blank
S = titration volume of sample
N = normality of sodium thiosulfate solution

PRECISION:

For samples analyzed in triplicate, the relative standard deviation is generally less than 5%.

NOTES

1. Store the saturated KI solution in the dark. Determine the concentration of the KI solution daily by adding 2 drops of starch solution to 0.5 ml of KI solution in 30 ml acetic acid-chloroform (3:2). If a blue color is formed which requires more than 1 drop of 0.1 N sodium thiosulfate solution to discharge, discard the iodide solution and prepare fresh. If the solution turns yellow, discard and prepare fresh also.

REFERENCES

1. AOCS. (1989) Peroxide value (CD 8 - 53, reapproved 1989). *In* Official Methods and Recommended Practices of the American Oil Chemists Society, fourth edition, D. Firestone (ed.). Am. Oil Chem. Soc., Champaign, IL, 2p.

2. Hara, S. And T. Yoichiro (1988). A highly sensitive method for the micro-determination a lipid hydroperoxides by potentiometry. J. Am. Oil Chemists' Soc. 65(12):1948-1950.

3. Van Dolah, F.M. and S.B. Galloway (eds.) (1988). Biomedical Test Materials Program: Analytical Methods for the Quality Assurance of Fish Oils, NOAA Tech. Mem. NMFS - SEFC - 211; 115p.

3.3 ALDEHYDES (AV)

INTRODUCTION

The *p*-anisidine value is a standard IUPAC method for the measurement of aldehydes (principally 2-alkenals) present as oxidation products in oils and fats (1). It is applicable to both animal and vegetable oils and fats. The standard IUPAC method is used by the BTM Program for the analysis of aldehydes in fish oil and esters of fish oil.

PRINCIPLE

P-anisidine reacts with aldehydic compounds in oils and fats in the presence of acetic acid to produce a yellowish product. The intensity of color of the product is measured by absorbance at 350 nm. The intensity depends not only on the amount of aldehydes present, but also on their structure. It has been found that a double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance four or five times. Thus the 2-alkenals contribute substantially to the value (1,2). The reaction between *p*-anisidine and aldehydes involves the formation of water. Hence, the presence of moisture in any of the reagents or in the sample leads to incomplete reaction and, consequently, low values. The *p*-anisidine value is defined by convention as 100 times the optical density, measured in a 1 cm cell, of a solution containing 1 g of the oil in 100 ml of a mixture of solvent and reagent.

APPARATUS

- 10-ml test tubes, with teflon-lined screw caps
- 25-ml volumetric flasks.
- Automatic positive displacement pipet (1 ml and 5 ml capacity)
- Spectrophotometer capable of measuring absorbance at 350 nm.
- Matched pair of 1 cm glass spectrophotometer cuvettes
- Analytical balance, accurate to $\pm$0.001 g
- Timer, 60 minute

REAGENTS

- Isooctane (ACS reagent grade)

- Glacial acetic acid, analytical reagent quality (Note 1)

- *p*-Anisidine (Sigma), 2.5 g/L in glacial acetic acid (or 0.25 g/100 ml acetic acid). Store in a refrigerator in the dark. This must be made up weekly (Note 2).

PREPARATION OF SAMPLES

1. The sample should be clear and dry. Particulate matter may be removed from the sample by filtration.

DETERMINATION

1. Weigh 0.5 - 4.0 g (to the nearest 0.001 g) oil or ester sample into a 25 ml volumetric flask (Use a glass pipet). Dissolve and dilute to volume with isooctane. A weight of 0.5 g is used for fish oils and esters.

2. Measure absorbance (A_b) of the sample solution at 350 nm with the reference cell filled with isooctane.

3. Pipette 5.0 ml of the oil solution into a test tube. Pipette 5.0 ml of isooctane into a second test tube (solvent blank). Add 1.0 ml of the *p*-anisidine solution to each tube. Shake.

4. Incubate the tubes at room temperature in the dark exactly 10 minutes. Measure the absorbance (A_s) of the solution in the sample tube at 350 nm, using the solvent blank in the reference cell.

CALCULATION

The anisidine value (AV) is given by the formula:

$$AV = 25 \times \left[\frac{1.2\ (As) - Ab}{m}\right]$$

where,

A_s = the absorbance of the oil solution after reaction with the *p*-anisidine reagent;

A_b = the absorbance of the unreacted sample;

m = the mass, in g, of the sample.

PRECISION

Duplicate analyses generally display less than 5% relative standard deviation.

NOTES

1. Since glacial acetic acid is highly hygroscopic, it is essential to check its moisture content by a Karl Fischer determination (section 6 in this manual). If the content exceeds 0.1 percent, the acetic acid must be discarded.

2. *P*-anisidine solutions having an absorbance greater than 0.200 when measured in a 1 cm cell at 350 nm against isooctane as a blank should be discarded.

REFERENCES

1. IUPAC. (1979) Determination of the *p*-anisidine value (p-AV) (#2.504). Pp. 143-144. *In* Standard Methods for the Analysis of Oils, Fats and Derivatives, sixth edition, C. Paquot (ed.). Pergamon Press, NY, 170 p.

2. IAFMM. (1981) Recommended method of analysis for determination of anisidine value of fish oil. *In* Fish Oil Bull. No. 8, June, 2p.

4.0 ORGANICS

4.1 PCBs AND PESTICIDES

INTRODUCTION

Neither AOCS nor AOAC has an official method for the determination of PCBs or pesticides in marine oils. There are first and final action packed column gas chromatographic methods (1,2) for the determination of organochlorine pesticides and polychlorinated biphenyl residues in fish and non-fatty foods. There is also a gel permeation first action method (3) for organochlorine residues in poultry, beef, and swine fats. The method developed by the BTM Program for the determination of PCBs and pesticides in oils is an adaptation of methods employed by the EPA (4) using capillary column gas chromatography and electron capture detection.

PRINCIPLE

The determination of PCBs and pesticides is preceded by the separation of these classes of compounds from other chemical components in the marine oil being analyzed. Gel permeation chromatography is first utilized to separate the PCB/pesticide fractions from the oil on the basis of molecular weight. Florisil adsorption chromatography is then used to separate the PCB/pesticide fractions from other minor components of similar molecular weight in the oil. The sample is then chromatographed simultaneously on two capillary GC columns of different polarity, which serves to confirm identification and quantitation of pesticide and PCB components. The two columns are installed in the same injection port of the gas chromatograph, but each is plumbed to a different electron capture (EC) detector and integrator. PCBs and pesticides are identified by comparison of the retention times of sample components to those in external standards containing a mixture of pesticides or Arochlor 1254. Pesticides are individually quantitated by a microprocessor associated with the gas chromatograph. For quantitation of PCBs, the peak areas for five representative peaks in the sample and the 1254 standard are compared and reported as total PCBs based on Archlor 1254 (5, 6).

APPARATUS

- Column for gel permeation chromatography, 25 cm x 2.5 cm (Kontes)
- HPLC pump (Laboratory Data Control)
- Rotary evaporator (Brinkmann Buchi RE120 or equivalent)
- Aspirator pump (Cole-Parmer)

- Column for florisil chromatography; 10 cm x 1.0 cm (Tudor)
- Gas chromatograph (Hewlett Packard 5880A or equivalent), equipped with two capillary columns installed in one injection port and plumbed to individual electron capture detectors (EC), two electronic integrators, and an autosampler
- Columns: DB5, 0.25µm film thickness, 30m length (J&W Scientific) and SPB608, 0.25µm film thickness, 30m length (Supelco)
- Rotating sample mixer (Fisher-Kendall or equivalent)
- Analytical balance capable of weighing to ± 0.0001 g (Mettler AE200 or equivalent)
- Modified 100 ml pear-shaped flask, with 1 ml volumetric tube (Figure 4.1-1) (custom-ordered, Southeastern Laboratory Apparatus.

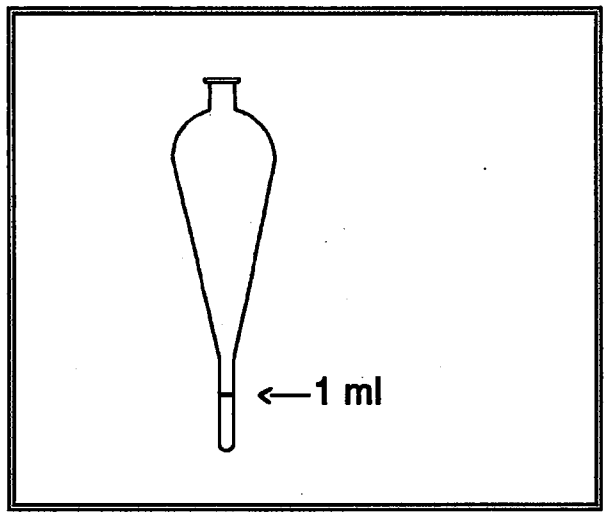

FIGURE 4.1-1. Modified Pear Flask

- Hydrocarbon trap (Chrompak)
- Oxygen trap (Chrompak)
- Moisture trap (Chrompak)
- Autosampler vials (Hewlette-Packard)
- Vortex mixer

REAGENTS

- Biobeads SX-3 (Biorad)
- Methylene chloride:cyclohexane (50:50) (Organic residue analysis grade)
- Isooctane (Organic residue analysis grade)
- Florisil, 60/100 mesh (Supelco)
- Petroleum ether:ethyl ether (80:20) (Organic residue analysis grade)
- Na_2SO_4, anhydrous (Baker)
- Hexane, pesticide grade (Organic residue analysis grade) *not used routinely*
- Hydrogen, ultra-high purity
- Nitrogen, ultra-high purity
- Air, breathing quality

PREPARATION OF STANDARDS & SAMPLES

I. Standard Preparation

1. Arochlor 1254 PCB standard: Nanogen Analytical Standards
2. Mixed Pesticide Standard: Nanogen Analytical Standards
3. Mixed Pesticide Standard: Individual standards received from Nanogen Analytical Standards are in toluene and are at known concentrations. The working standard is made up from the individual standards using isooctane as the solvent. A 250 ml stock solution is made up and stored at -30°C and a 25 ml working standard is aliquoted from this stock solution. The standard is then aliquoted into 100 µl auto-sampler vials with teflon-lined crimp tops for GC analysis.
4. PCB Standard: PCB Arochlor 1254 is received from Nanogen Analytical Standards. The standard is in toluene at a known concentration and is diluted quantitatively to yield 25 ml of working standard with a final concentration of 0.08 ng/µl.

TABLE 4.1-1. Composition of Mixed Pesticide Standard

Name	ng/*u*l
a-BHC	0.090
HCB	0.090
bBHC	0.090
Lindane	0.090
Heptachlor	0.090
a-chlordene	0.090
aldrin	0.090
hept EPX	0.090
g-chlordane	0.090
o,p-DDE	0.090
a-chlordane	0.090
transnanochlor	0.090
dieldrin	0.090
endrin	0.090
p,p-DDE	0.090
o,p-DDD	0.090
p,p-DDD	0.090
o,p-DDT	0.090
p,p-DDT	0.090

II. Sample Preparation

1. Mix oil thoroughly on sample mixer.

2. Weigh duplicate samples, between 1.5 - 2.5 g, to the nearest 0.0001 g, into individual 25 ml volumetric flasks.

3. Bring each sample to volume with 50:50 methylene chloride:cyclohexane and mix well with a vortex mixer.

III. Sample Clean-up

Gel permeation: Gel permeation chromatography is performed on 25 cm x 2.5 cm column of Biobeads SX-3 eluted with 50:50 MeCl:cyclohexane. A closed system is used (Figure 4.1-2) in which the solvent is pumped onto the column with an HPLC pump and sample is introduced via a sample injection valve with a 1 ml sample loop of teflon tubing. Eluent from the column is directed either to a waste jar, to a precalibrated pre-run catch flask (which allows collection of a known volume of eluent before sample collection begins), or to a sample flask.

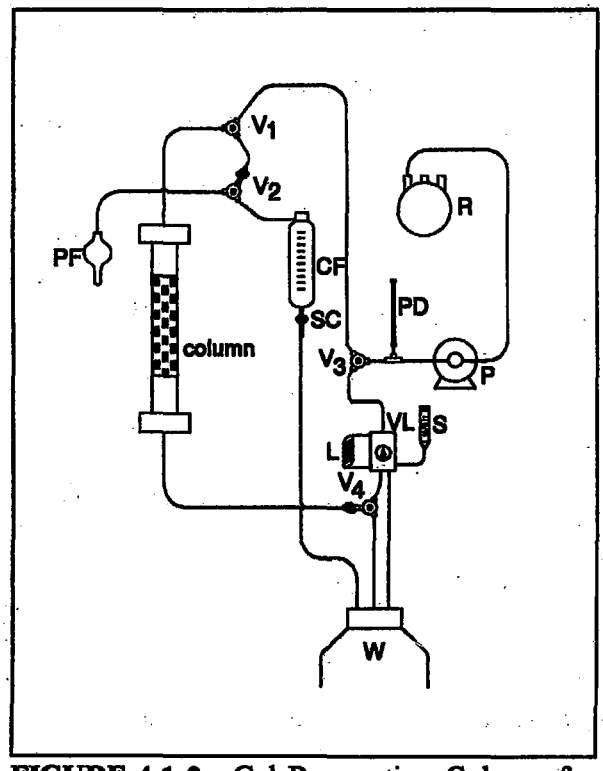

FIGURE 4.1-2. Gel Permeation Column for Pesticide/PCB Sample Cleanup

1. Fill the reservoir (R) with eluting solvent. Open the bottom valve (v4) and run the pump (P) for 30 min or until the column packing is totally moistened with solvent.

2. To apply sample to the column, fill the sample syringe (S) with 5 ml of sample. With the loop valve (vL) in "load" position, allow 4 ml of sample to flow through the loop (L) and into the waste bottle (W). Switch the loop valve to "column" position, to direct the 1 ml content of the loop onto the column, while simultaneously closing the stopcock (SC).

3. The pre-run is carried out for 11 minutes at a flow rate of 4.9 ml/min. Once the forerun has eluted, switch valve 2 (v2) to direct the eluting solvent to a pear shaped flask (PF). Elute for 23 minutes at a flow rate of 4.9 ml/min. Upon completion of the elution, switch the outlet valve (v2) back to its original position, and open stopcock (SC).

4. After sample is eluted, back-flush the column to prepare it for the next sample. Turn pump off and wait until pulse damper (PD) is empty. Then turn valves 1,3, and 4 to reverse flow of the solvent through the column. Turn pump on and run for 5-10 min. Store column with valve 4 in the closed position to keep eluting solvent on the column after all valves are returned to original position.

Rotary evaporation: Evaporate the eluting solvent to 0.5 ml in the pear-shaped flask using an aspirator pump as the source of vacuum. Be certain the sample does not go to complete dryness. Water bath temperature should be 40°C and a spin rate setting of 5. Add an additional 0.5 ml of isooctane and continue evaporation to a volume of 0.5 ml. Repeat the last process 3 times. Following the last evaporation, bring volume to 1 ml with isooctane and cap the sample.

Florisil chromatography: Fill a 10 cm x 1.0 cm column with florisil to 7.8 cm and activate the column overnight in a 130°C oven. Cool the column in a desiccator, place it in an appropriate holder, and add 1.0 cm of hexane-extracted Na_2SO_4 to the top of the florisil (Note 1). Wet the column packing with 20 ml petroleum ether:ethyl ether (80:20). Do not allow the column packing to go dry; keep the solvent level at the top of the Na_2SO_4 layer by closing the stopcock when the solvent is about 1 cm above the Na_2SO_4. Transfer the 1 ml sample from the pear-flask to the column using a disposable glass Pasteur pipet. Open the stopcock to begin elution of the sample. Follow the transfer with two 1 ml washes of the sample flask with petroleum ether:ethyl ether (80:20). When the added sample on the column reaches the top of the Na_2SO_4 layer, add an additional 5 ml of petroleum ether:ethyl ether (80:20) to wash the walls of the column. Following sample application, elute with the appropriate volume (determined as described in Note 2). The column flow should be 4-5 ml/min; it may be necessary to pressurize with nitrogen to attain this flow rate. The eluent is collected in a modified pear-flask (FIGURE 4.1-1).

Rotary evaporation: Add 1 ml of isooctane to the eluent from the florisil column. Evaporate to a volume of 1.0 ml, as indicated by the calibrated tip of the pear-shape flask. Transfer approximately 0.1 ml to a 100µl glass autosampler vial, and seal with a

crimp cap, fitted with a teflon-lined septum, for GC analysis. The remainding sample is stored in a 2 ml glass crimp-top vial at -20°C.

DETERMINATION

Samples are chromatographed simultaneously on two capillary GC columns of different polarity installed into the same injection port, using a two-hole ferrule. The DB-5 column is used for quantitation. The SPB608 column is used for confirmation of peak identifications and quantitations. Since quantitation is based on external standards, the use of an autosampler is recommended to obtain the necessary precision in sample volume. Temperature programming parameters used are listed in TABLE 4.1-2. The flow rate is set manually at about 1 ml/min. The carrier gas is ultra high purity hydrogen, which is further cleaned by in-line moisture, oxygen and hydrocarbon traps.

TABLE 4.1-2. GC Temperature Program Parameters for the Separation of PCB/Pesticide Fractions

Detector temperature	300°C
Injection temperature	250°C
Oven temperature profile	
initial value	90°C
initial time	0.50 min
level 1	
program rate	5.00°C/min
final value	165°C
final time	0.00 min
level 2	
program rate	0.10°C/min
final value	172°C
final time	0.00 min
level 3	
program rate	1.00°C/min
final value	220°C
final time	60.00 min
post value	230°C
post time	15.00 min

1. Sample vials are placed in an autosampler in the odd-numbered sample slots. The even-numbered sample slots contain wash vials filled with isooctane. A PCB and a pesticide standard, of known composition, are run after every two samples. The pesticide standard is used to automatically up-date the calibration file for peak retention time and response. The sample injection volume is 2µl.

2. To program HP5880 gas chromatographic runs, the analyst enters an "identification code" and a "multiplier" for each sample into a "Sample Table." When a vial slot is identified as a standard, the instrument microprocessor invokes an automatic recalibration of response factors for each pesticide.

CALCULATION

1. **Pesticides**: Calibration files, created and stored in microprocseeor memory by the analyst, are automatically up-dated after each standard run. For sample runs, identification for each pesticides is achieved automatically by comparison of sample peak retention times with those contained in the calibration file for known pesticides. The amount of each identified component is automatically calculated by the equation given below. The multiplier takes into account the original sample weight and the volume chromatographed.

$$ppm\ unknown = \frac{peak\ area\ (sample)}{peak\ area\ (std)}\ x\ ng\ std\ x\ multipler$$

where,

$$multipler = \frac{1}{2\ \mu l}\ x\ \frac{25\ ml}{sample\ wt}\ x\ \frac{1}{1.27}$$

The sample volume chromatographed is 2 µl, 25 ml is the original sample volume, and 1.27 is a correction factor correcting for the fact that 1.27 ml of the original sample is actually applied to the gel permeation column in the first step of sample clean-up, rather than 1 ml in the sample loop, due to the volume of tubing leading to the column. This correction factor must be determined in each individual laboratory.

The analyst must confirm the peak identifications by comparing the identifications made by the microprocessor for separation on the DB-5 chromatogram with those made for separation on the confirmation column. If a component identified as a pesticide in the DB-5 chromatogram is missing in the SPB608 chromatogram, the identification is considered an error.

PCBs: Identification and quantification of PCB components are made by the analyst. Peak areas for five representative peaks in the sample and the 1254 standard are compared. The equation given below is used to calculated total PCBs based on Archlor 1254 (5, 6).

$$ppm\ total\ PCBs = \frac{summed\ peak\ area\ (sample)}{summed\ peak\ area\ (std)}\ x\ ng\ std\ x\ multiplier$$

where,

$$multipler = \frac{1}{2\ \mu l}\ x\ \frac{25\ ml}{sample\ wt}\ x\ \frac{1}{1.27}$$

Two µl is the sample volume chromatographed, 25 ml is the original sample volume, and 1.27 is a correction factor correcting for the fact that 1.27 ml of the original sample is actually applied to the gel permeation column in the first step of sample clean-up, rather than 1 ml in the sample loop, due to the volume of tubing leading to the column.

PRECISION

Duplicate samples of oil carried independently through the sample preparation procedure and gas chromatography analysis should have a relative standard deviation of less than 15%.

NOTES

1. Cleaning of Na_2SO_4 by extraction with hexane in a Soxhlet extraction apparatus: Place approximately 100 g Na_2SO_4 in pyrex soxhlet thimble. Place the thimble in the soxhlet apparatus. The reflux should be cooled by running cold water in the jacket. Place boiling chips and 300 ml hexane in a round botton flask and attach to the bottom. After refluxing overnight, place dry Na_2SO_4 in an oven at 130°C. Store in oven until use. Before use in the florisil column, allow the Na_2SO_4 to come to room temperature in a desiccator.

2. Calibration of florisil by lot: Because each lot of florisil varies in its adsorbent activity, each new lot of florisil must be calibrated to determine the volume of eluting solvent required to remove the PCBs and pesticides. Prepare a florisil column as in the sample clean-up procedure above. Elute a 10-fold concentrated PCB and pesticide standard, collecting three 10-ml fractions followed by 20 2-ml fractions. Analyze each fraction for PCBs and pesticides by GC as described above. Determine the elution volume where standards are no longer present. This determines the elution volume used in the florisil chromatography step above.

3. When setting up the gel permeation column the analyst must verify the fore-run and collection cutoffs. Collecting fractions on the fore-run and subsequent GC determination will indicate when the analytes of interest start eluting from the column. The amount of solvent and time of collection run is also determined by GC analysis of fractions.

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4.2 UREA

INTRODUCTION

The AOAC has several classical tests for urea including the Urease Test for Urea (#44.164)(1) and the Xanthydol Test for Urea (#44.165) (2). These tests usually require an analyst skilled in microcrystal testing. None of the methods are directly applicable to the estimate of urea residues in fatty acid esters. The method used by the BTM Program is a modification of a spectrophotometric method developed for the determination of suspected urea in urine stains on foods and containers (3). This method was chosen because it is sensitive at the ppm level, does not require an analyst skilled in microcrystal testing, and is based on an initial extraction of the material to be tested.

PRINCIPLE

Urea is extracted from esters of fish oil into distilled water. The urea assay is performed on the water extract. The procedure is based on the formation of ammonia from the reaction of urea with urease followed by the formation of a blue compound, indophenol, by the reaction of ammonia and phenol in the presence of hypochlorite. Indophenol is quantified by spectrophotometry. A calibration curve is constructed using urea standards of several concentrations versus their absorbance at 625 nm. The concentration of urea in samples is determined by linear regression analysis using the calibration curve constructed.

APPARATUS

- Culture tubes, 125 mm x 25 mm
- Automated pipet, positive displacement, 1 ml
- Counter top centrifuge (capable of attaining 1000 x g)
- Spectrophotometer (capable of measuring $\pm$ 0.001 Abs units)
- Glass cuvettes, 1 cm
- Volumetric tubes, 10 ml
- Vortex mixer

REAGENTS

- Chloroform (Reagent grade)

- Urease (Sigma), 10 mg/ml in distilled water
- Manganous sulfate, reagent grade, 0.05%
- Urea, reagent grade, 1.0 g/L acetone stock solution
- Hypochlorous acid: add 5 ml 5.25% commercial bleach to 20 ml distilled H_2O. Adjust to pH 6.5-7.0 with HCl.
- Phenol solution: 0.5 g NaOH and 2 g phenol in 20 ml distilled H_20

PREPARATION OF STANDARDS & SAMPLES

All samples, procedural blanks and spiked samples are prepared and analyzed in duplicate. The procedural blank (Note 1) is an empty tube that is carried throughout the entire procedure. The spiked sample (Note 2) is included to determine the % recovery from the extraction procedure.

I. **Extraction of urea from ethyl esters of fish oil:**

1. Weigh 0.5 g esters into a culture tube.
2. Add 3 ml $CHCl_3$.
3. Add 1 ml distilled H_2O.
4. Extract by mixing on Vortex mixer 15 sec.
5. Centrifuge tube 15 min at 1000 x g.
6. Remove water layer and transfer to 10 ml volumetric centrifuge tube.
7. Repeat extraction procedure (3-6) two more times.
8. Back-wash the combined water extracts with 1 ml $CHCl_3$ by mixing on Vortex 15 sec, centrifuging 15 min at 1000 x g and removing the $CHCl_3$ layer.
9. Take the combined water layers to 10 ml in a volumetric tube.

II. **Preparation of Standard Curve**

1. The assay is linear from 3-15µg urea/ml. A standard curve of 0, 3, 6, 9, 12, and 15µg/ml urea is prepared from the urea stock solution described above by pipetting 0, 30, 60, 90, 120, and 150µl of the stock solution into 10 ml volumetric tubes, evaporating the solution to dryness under nitrogen, and bringing to a volume of 10 ml with H_2O.

DETERMINATION

1. To each standard, blank and sample tube, add 3 drops urease solution.
2. Heat in hot water bath at 55°C for 30 min.
3. Cool to room temperature.
4. Add 1 drop $MnSO_4$.
5. Add 10 drops hypochlorous acid solution.
6. Immediately add 12 drops phenol solution. Incubate at room temperature for 10 minutes.
7. Transfer 1 ml of each solution to 1 cm cuvette and read absorbance at 625 nm.

CALCULATION

Create a standard curve of urea concentration versus absorbance. Determine the concentration of urea in the unknown samples by linear regression using the standard curve constructed. Samples must be diluted to a concentration that falls within the linear range of the standard curve.

PRECISION

Samples analyzed in duplicate should have less than a 10% relative standard deviation.

NOTES

1. Color intensity is affected by age of the reagents; therefore, a procedural blank and/or control should be carried throughout the entire procedure. The procedural blank consists of an empty tube which is carried through all extraction and determination steps as a sample.

REFERENCES

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4.3 ANTIOXIDANTS

4.3.1. TERT-BUTYL HYDROQUINONE (TBHQ)

INTRODUCTION

The BTM Program uses the antioxidant tertiary butyl hydroquinone (TBHQ) in many of the test materials produced at the Charleston Laboratory. The FDA set a legal limit for the concentration of TBHQ in food products at 0.02%. A published method for simultaneous analysis of TBHQ, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) in vegetable oils (1) was adapted for the determination of TBHQ in fish oils. The method was further modified, by the BTM Program, for the determination of TBHQ in ethyl esters of fish oils (2).

PRINCIPLE

For analysis of fish oil, TBHQ is extracted into acetonitrile containing an internal standard, propyl paraben (propyl parahydroxybenzoate). In order to stabilize TBHQ in the reduced hydroquinone form, silyl derivatives are prepared. The derivatized components, in acetonitrile, are analyzed by GLC with flame ionization detection. Separation is achieved on a megabore wall-coated open-tubular (WCOT) column. The amount of TBHQ is calculated as a single-point ratio of the peak area to that of the internal standard. Unlike the triacylglycerols, ethyl esters are quite soluble in acetonitrile. For the determination of TBHQ in ethyl esters, the TBHQ and esters are solubilized in acetonitrile containing an internal standard and silyl derivative prepared. Both TBHQ and the internal standard elute prior to the esters during GLC analysis. Following elution of these components, the oven temperature is increased ballistically to rapidly elute ethyl esters.

APPARATUS

- Volumetric flasks , 50 ml.
- Automated positive displacement pipet, 0.2-1.0 ml (SMI or equivalent).
- 16 mm x 125 mm test tubes with teflon-lined screw caps.
- Gas chromatograph equipped with flame ionization detector and electronic integrator (HP5890 or equivalent).
- Analytical balance, accurate to ±0.001 g (Mettler AE200 or equivalent).
- Megabore WCOT column, 0.53 mm (J & W Scientific. DB-5 or equivalent).
- Hamilton syringe, 10µl (or equivalent)

REAGENTS

- Acetonitrile (ACS reagent grade).
- Propyl paraben (Sigma), 200µg/ml in acetonitrile.
- Tertiary butyl hydroquinone (Kodak), 200µg/ml in acetonitrile.
- N,O-bis-(trimethylsilyl) trifluoroacetamide, BSTFA (Pierce).

PREPARATION OF STANDARDS & SAMPLES

I. **Calibration Standard:**

Add 1 ml propyl paraben stock solution and 1 ml acetonitrile to 1 ml TBHQ stock solution. This standard is used to calculate a GC response factor for TBHQ.

II. **Sample preparation for ethyl esters:**

1.. Weight 0.1 g ethyl ester (to the nearest .001g) into a 16 x 125 mm tube.

2. Add 1 ml aliquot of acetonitrile.

3. Add 100µl of the internal standard propyl paraben (200 µg/ml).

III. **Sample preparation for oil:**

1. Weigh 1 g oil (to the nearest 0.001 g) into a 16mm x 125 mm tube.

2. Add 1 ml of 200 µg/ml propyl paraben in acetonitrile.

3. Shake by hand for 15 sec.

4. Centrifuge in counter top centrifuge 3 minutes to separate phases.

5. Remove acetonitrile layer to a second tube.

6. Add 1 ml acetonitrile (not containing propyl paraben).

7. Shake for 15 seconds.

8. Centrifuge for 3 minutes.

9. Remove acetonitrile layer and put in another tube.

10. Repeat extraction procedure once (6-9).

IV. **Silyl derivatization:**

1. To each extracted sample and the calibration standard add 0.25 ml BSTFA.
2. Vortex samples for 1 min. and allow to stand at room temperature for 15 min.

DETERMINATION

The following parameters are used for analysis of the derivatized samples by gas chromatography (TABLE 4.3.1-1).

TABLE 4.3.1-1. GC Parameters for the Determination of TBHQ

Injection temperature	250°C
FID temperature	250°C
Oven temperature	
Initial temp	150°C
Initial time	28.00 min
Rate	40°C
Final temp	250°C
Final time	40.0°C
Stop	70.5 min
Flow Rate	30 ml/min

1. Make injections of 1µl derivatized calibration standard or sample using a 10µl syringe. Analyses are performed in duplicate.

CALCULATION

1. **FID Response Factor:** The relative response factor for TBHQ is calculated by microprocessor using the following equation:

$$Response\ factor\ (TBHQ) = \frac{amount\ sample}{area\ sample} x \frac{area\ internal\ standard}{amount\ internal\ standard}$$

The known weights of TBHQ and the internal standard are entered by the analyst. The response factor for the internal standard is defined as 1. This calibration is run in duplicate.

2. **Percent TBHQ**: The amount of TBHQ in a sample is calculated :

$$\%\ TBHQ = \frac{area\ (sample)\ x\ RF\ (sample)}{area\ (IS)\ x\ RF\ (IS)} x \frac{wt\ (IS)}{wt\ (sample)} x\ 100$$

RF = response factor
IS = internal standard

PRECISION

Duplicate analyses on a sample should have a relative standard deviation of less than 3%.

REFERENCES

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4.3.2 TOCOPHEROLS

INTRODUCTION

Both α- and γ-tocopherols are routinely added to many of the BTM Program test materials for their antioxidant capacity. The test materials containing tocopherols are analyzed to assure the levels added. Levels of α- and γ-tocopherols are determined in placebo vegetable oils to provide researchers with information on naturally occurring quantities present or quantities added when test materials and placebos are matched for antioxidant content.

Official methods for gas chromatographic (1) and colorimetric (2) determination of α-tocopherol have received final action by the AOAC. Neither method provides for the determination of γ-tocopherol. The method used by the BTM Program for fish oils and esters of fish oils is a modification of a method developed at the USDA Human Nutrition Research Center in Beltsville, MD (3). This method permits determination of cholesterol, major plant sterols, and α- and γ-tocopherols from a single analysis.

PRINCIPLE

A sample containing approximately 0.1 g of lipid (fat, oil or lipid extract) is mixed with a known weight of the internal standard (5-α-cholestane) and saponified at 80°C with aqueous KOH in the presence of pyrogallol. The non-saponifiables are extracted with cyclohexane, the solvent is removed under nitrogen, and trimethylsilyl ethers are prepared using N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane in pyridine. This derivatized, total non-saponifiable fraction is chromatographed on a non-polar fused silica wall-coated open-tubular (WCOT) column. The α-and γ-tocopherol in sample chromatograms are identified by comparison of their peak retention times (relative to that of the internal standard) with those of the tocopherols in standard chromatograms. For routine analyses, a single point ratio is used to calculate the amounts of α- and γ-tocopherol.

The analysis of a reference material accompanies each group of samples analyzed to ensure accuracy of analysis. Regular participation in collaborative analyses, such as the Smalley Check Sample Program (AOCS, Champaign, IL), provides continual evaluation of analyst and instrumentation.

APPARATUS

- Gas chromatograph (HP5890 or equivalent), equipped for use with capillary columns (0.22-0.32 mm id), with an FID detector and optional autosampler

- Non-polar flexible fused-silica capillary column, 25 m x 0.2 mm x 0.3μ film thickness (Hewlett-Packard Ultra 2) or 60 m x 0.22 mm x 0.25μ film thickness (J&W Scientific DB-1)

- Centrifuge (Centra 4 International Centrifuge or equivalent) with rotor to accommodate 25 x 125 mm screw cap tubes and operate at 1300 rpm
- Nitrogen evaporator with water bath capable of 37°C (Meyers N-Evap or equivalent)
- Water bath, 80°C (Precision Scientific or equivalent)
- Vortex mixer
- Pasteur pipettes, disposable
- Automatic positive displacement pipettor capable of delivering 1 ml ±0.01 ml (Eppendorf Repeater Pipet Model No. 2226 000-6 or equivalent)
- Centrifuge tubes, 25 x 125 mm, with teflon lined screw caps
- Silanized, borosilicate glass tubes, 16 x 125 mm, with teflon lined screw caps,
- Analytical balance (Mettler AE163 or equivalent)
- Mechanical sample mixer (Fisher-Kendall or equivalent)
- Moisture trap (Chrompak)
- Oxygen trap (Chrompak)
- Hamilton syringe, 10µl (or equivalent)
- Volumetric flasks, 100 ml
- Glass transfer pipettes, 10 ml
- Solvent/reagent bottles with

REAGENTS

- Hexane, ACS reagent grade
- Isooctane, ACS reagent grade
- Butylated hydroxytoluene (BHT), reagent grade
- Nitrogen, compressed
- Potassium hydroxide solution - dissolve 30 g KOH (85%) in 20 ml distilled water.)

- Pyridine, silation grade (Pierce Chemical Co.)
- N,O-bis-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (SupelcoSylon BFT or equivalent). Sylon BFT is corrosive and will attack syringe needles and plungers, thus they should be rinsed promptly. Pryidine will cause some elastomers to swell (e.g., vitons). Glass and teflon are resistant to pyridine.
- Sylon CT (Supelco)
- Cholesterol standard (Eastman Kodak)
- 5-α-Cholestane (Supelco)
- Absolute ethanol, reagent grade, degassed under vacuum using a rotary evaporator.
- Pyrogallol, reagent grade (Malinckrodt), 3% w/v in degassed (under vacuum using a rotary evaporator) absolute ethanol.
- Helium (99.999% pure), carrier gas
- Hydrogen (99.99% pure), FID
- Compressed air (breathing quality or better), FID
- Nitrogen, compressed, make-up gas
- Cyclohexane, degassed under vacuum using a rotary evaporator
- Reference sample - soft-gel encaps;ulated fish oil containing added α- and γ-tocopherols (Charleston BTM PRogram)

PREPARATION OF STANDARDS AND SAMPLES

Tocopherols can be destroyed by sunlight and white fluorescent light, especially under alkaline conditions. This method is carried out under yellow fluorescent lighting. Subdued incandescent lighting may be used if all windows are covered to exclude sunlight.

I. STANDARDS

1. **Internal Standard**: Accurately weigh approximately 50 mg of 5-*a*-cholestane and 20 mg of BHT, transfer to a 100 ml volumetric flask and bring to volume with isooctane. Transfer 10 ml of this stock solution to a 100 ml volumetric flask and dilute to volume to give a working standard with a concentration of 40-50μg/ml 5-*a*-cholestane + 20μg/ml BHT.

2. **Primary Reference Standard**: Prepare stock solutions of both α- and γ-tocopherol as follows: Accurately weigh approximately 50 mg of tocopherol, transfer to a 100 ml volumetric flask and bring to volume with isooctane. Transfer 10 ml of this stock solution to a 100 ml volumetric flask and dilute to volume to give a working standard with a concentration of 40-50μg/ml. Pipet 1.0 ml of the working standard and 1.0 ml of the internal standard into a derivatization tube and proceed as outlined in "preparation of samples", beginning with step 3. The volumes of tocopherols should be adjusted, as required, to keep the concentration in line with that expected in the samples.

3. **Secondary Reference Material:** Prepare as specified for samples, starting with Step 1.

II. SAMPLES

1. Weigh 80-100 mg of oil or ester concentrate (to the nearest 0.0001 g) into a 50 ml screw capped centrifuge tube.

2. Add 1.0 ml internal standard to each sample.

3. Place the tube in a 37°C water bath and evaporate the solvent under a gentle stream of dry nitrogen (N-Evap).

4. Add 8 ml pyrogallol-absolute ethanol solution and 0.5 ml saturated KOH.

5. Cap tubes tightly and vortex 5 sec.

6. Heat tubes for 8 min at 80°C in a heated waterbath; shake vigorously after 1, 2 and 4 min so that the solution washes tube sides and caps.

7. Remove tubes and cool in running tap water for 15 sec.

8. Add 10 ml cyclohexane and vortex for 5 sec.

9. Add 6 ml distilled water and mix for 15 min (mechanical mixer).

10. Centrifuge for 15 min (1300 x g).

11. Transfer cyclohexane layer to a 16mm x 125 mm silanized culture tube.

12. Place tubes in N-Evap water bath at 37°C and evaporate the cyclohexane under a gentle stream of nitrogen.

13. Add 100μl pyridine and 100μl Sylon BFT; vortex 5 sec and allow to stand 15 min at room temperature. Transfer sample to 200 μl autosample vial for GLC.

DETERMINATION

The prepared samples and standards are chromatographed isothermally on a fused silica capillary column (DB-1 or HP Ultra 2) using an all-glass split injection port with a split ratio of approximately 1:40. Carrier gas is helium (99.99% pure) with in-line moisture and oxygen traps. The average linear velocity is approximately 30 cm/sec. Nitrogen is used as the auxillary gas to the flame ionization detector (FID). The combined flow rate at the detector (carrier + auxillary) is 30 ml/min. The following temperature parameters are used on the HP5890 GC: injection port - 300°C; detector - 300°C; column oven - 280°C.

A 1 µl sample is injected, either manually using a 10µl syringe, or by autosampler.

CALCULATION

Most GC systems currently in use are capable of automated peak identification based on a retention time window, and quantitation by an internal standard method using either single-point or multiple-point ratios. Follow manufacturers guidelines for the set-up of these procedures. Procedures for manual calculations follow:

Relative retention times are calculated for the tocopherol standards versus that of the internal standard (Equation I). The tocopherol peaks are then identified in the sample by comparison of the relative retention time of sample components with those of the tocopherol standards.

A single-point ratio is used for calculation of α- and γ-tocopherols in routine analyses. (Equations II and III). However, linearity should be confirmed for the range of 0.5 - 1.5 times the amount expected in the samples.

EQUATION I.

$$RRT_T = \frac{(RT_T - RT_S)}{(RT_C - RT_S)}$$

RRT_T = relative retention time for tocopherol
RT_C = retention time of cholestane
RT_T = retention time of tocopherol
RT_S = retention time of solvent

EQUATION II.

$$K_T = \frac{A_C \times W_T}{W_C \times A_T}$$

K_T = correction factor for tocopherol
A_C = area of cholestane in reference chromatogram
W_T = weight of cholestane in reference chromatogram
A_T = area of tocopherol in reference chromatogram
W_C = weight of tocopherol in reference chromatogram

EQUATION III.

$$T = \frac{A_T \times K_T \times W_C}{A_C \times W_S}$$

T = amount of α/γ in mg/g
A_T = area of tocopherol in the sample chromatogram
K_T = correction factor for tocopherol
W_C = weight of cholestane (μg) in sample chromatogram
A_C = area of cholestane in sample chromatogram
W_S = sample weight (mg)

PRECISION

The analysis is precise at $\pm$ 2% for duplicates.

REFERENCES

1. AOAC. (1984) Vitamin E in drugs, gas chromatographic method. (#43.152-43.159), Pp. 860-861. *In* Official Methods of Analysis of the AOAC, 14th edition, S. Williams (ed.) Assoc. Ofc. Anal. Chem. Arlington, VA, 1141 p.

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5.0 METALS

INTRODUCTION

The BTM Program performs analyses for a wide array of metals in the fish oil test materials produced by the BTM Program. The metals selected for analysis are those that may either be native to the oil or introduced during processing. These include the potentially toxic metals (Hg, Cd, As, Se, Pb, Ni, and Cr) and elements of nutritional importance and/or oxidation catalyst in biological test material (including Cu, Fe, and Zn). The methods for conducting the analyses were selected based on the need for time efficiency, both in terms of the time required for sample preparation and for analysis. The methods employed do not necessarily provide the lowest possible detection limit, but provide detection limits well below FDA or international regulatory limits imposed on fish and fishery products. Hg is the only metal regulated in fishery products by the FDA, with a limit of 1.0 ppm as CH_3Hg (methyl mercury). Other countries impose various limits on As, Cd, Cr, Cu, Hg, Pb, Sn, Se, and Zn contents of fishery products, generally above 1 ppm, but occasionally as low as 0.1 ppm for As, Cd, and Hg.

Standard methods are not currently available for the determination of metals in fish oils. The AOCS published a tentative method for the analysis of Cr, Cu, Fe, Ni, and Mn in vegetable oils using methyl isobutyl ketone (MIBK) extraction and analysis by flame atomic absorption spectrophotometry (1, Section 5.1) and graphite furnace (3, Secton 5.1). The MIBK extraction coupled with flame AA is sensitive only to 2.0 ppm of each metal and therefore not suitable for our purposes. The BTM Program utilizes microwave digestion of oil samples followed by analysis using either flame AA, hydride generation, or graphite furnace.

5.1 DIGESTION PROCEDURES

INTRODUCTION

A number of wet digestion methods are available for removing organic components from samples of biological origin (3,4). Oil samples present a particularly reactive matrix that make many of the conventional methods time-consuming and/or dangerous. The BTM Program employs a microwave digestion technique using closed teflon digestion vessels. This method is comparatively rapid, requiring several hours digestion time, as opposed to up to two weeks for digestion using conventional wet digestion techniques.

PRINCIPLE

The most commonly used methods for preparing biological matrices for metals analysis are a variety of wet acid digestion techniques (3,4). Most methods use a combination of nitric acid and another acid with stronger oxidizing power. Nitric acid readily reacts with both aromatic and aliphatic compounds. It rapidly oxidizes aliphatic polyhydroxyl compounds, degrading them to simple carboxylic acids (3). Nitric acid digestion of fish oils in open vessels yields an incomplete digestion, leaving a solid waxy residue even after an extensive digestion of 96 hours. This residue is believed to consist of paraffins which are the saturated products of the incomplete digestion. Complete digestion of fish oils using nitric/perchloric/sulfuric acids in micro Kjeldahl flasks can take up to two weeks. The digestion of samples in closed vessels improves the performance of the acids by increasing the temperatures achieved (5,6). Heating closed vessels by microwaves further enhances the performance of digestion by decreasing the time required to reach the desired temperatures. Microwave digestion techniques are relatively new; thus, no standard methods exist.

In the digest method using only nitric acid, developed at the Charleston Laboratory, fish oils or esters of fish oils are allowed to pre-digest in nitric acid at room temperature in open teflon microwave digestion vessels. The samples are then microwave digested with nitric acid in closed vessels for approximately three hours. The samples are allowed to cool, then transferred to volumetric flasks and diluted to 25 ml with distilled H_2O. This results in an incomplete digestion similar to that obtained after 96 hours of digestion in micro Kjeldahl flasks described above. For the nitric/sulfuric acid digestion method, a small amount of sulfuric acid is added and the samples microwaved until they char. Then nitric acid is added and the samples are allowed to pre-digest at room temperature overnight. The samples are microwave digested for approximately three hours, as in the previous method. The vessels are then opened, the contents evaporated to approximately 1 ml, and 30% hydrogen peroxide is added, 1 ml at a time, until a clear to very pale yellow digestate remains, with a volume of approximately 1 ml. The samples are then taken volumetrically to 10 ml with distilled H_2O. Two different digests are used because the first method involving nitric acid only gives better recoveries for As, Se, and Hg, and the nitric/sulfuric digest is a complete digest that gives good recoveries for the other elements.

The microwave digestion system consists of 120 ml teflon vessels with screw-on lids that possess a pressure release valve which opens at 120 psi. The vessels are capped using an automated "capping station" which achieves the same torque on all caps, ensuring that the release valve will open at the specified pressure. The vessels fit into a turntable and each vessel is vented into an overflow container. The microwave oven is equipped with an exhaust tube which is vented into a laboratory hood.

All sample digestions are accompanied by a blank, and a spiked fish oil or ester sample, in order to determine recovery and to ensure accuracy of the analyses.

APPARATUS

- Analytical balance (Mettler AE200 or equivalent)
- Automatic pipet (Rainin Pipetman 1000 or equivalent)
- Microwave digestion oven (CEM Corporation), equipped with vessel capping station, turntable and twelve closed teflon digestion vessels with pressure relief valves (Note 1)
- Volumetric flasks, 10 and 25 ml
- Nalgene bottles, 30 ml, acid washed

REAGENTS

- Nitric acid (Ultrex grade, J.T. Baker)
- Sulfuric acid (Ultrex grade, J.T. Baker)
- Hydrogen peroxide (Ultrex grade, J.T. Baker)
- Reference metal standards, 1000 ug/ml, for each metal of interest (Fisher or equivalent)
- Deionized H_2O (dH_2O)

PREPARATION OF STANDARDS AND SAMPLES

Proper protective clothing, gloves, and eye protection should be worn during sample preparation. Manufacturers safety instructions for use of the microwave digestion apparatus should be closely observed.

Nitric Acid-Only Digestion:

1. Weigh 1 g (to the nearest 0.0001 g) of fish oil or ester into a teflon digestion vessel. For the spiked oil/ester samples, add 0.1 ml of a mixed standard solution containing 100 µg/ml (ppm) for flame elements (Fe, Zn) and 10 µg/ml (ppm) for furnace elements (As, Cd, Cu, Cr, Pb, Hg, Ni, Se). For blanks, use an empty vessel. Each set of digest contains a blank and blank spike

2. Add 15 ml of HNO_3. Swirl.

3. Pre-digest overnight. Cover vessels loosely with lids.

4. Place lid on the teflon vessel and tighten the lid using the capping station to achieve proper torque.

5. Place all vessels (up to 12) in the turntable of the microwave oven. Being certain to connect vent tubes to the venting trap.

6. Begin heating the oven using three stages: 1) 30% power for 30 minutes; 2) 50% power for 60 minutes; and 3) 70% power for 100 minutes.

7. Remove the vessels from the oven and allow to cool to room temperature. A cool water bath can be used to speed the cooling process. After the vessels cool, vent them.

8. Remove the caps, using the capping station.

9. Transfer cooled digests to 25 ml volumetric flasks and take to volume with dH_2O. Transfer samples to 30 ml Nalgene bottles.

Nitric/Sulfuric Acid Digest:

1. Weigh 1 g (to the nearest 0.0001 g) of fish oil or ester into a teflon digestion vessel. For spiked oil/ester samples, add 0.1 ml of a mixed standard solution containing 100 µg/ml (ppm) for flame elements (Fe, Zn) and 10 µg/ml (ppm) for furnace elements (As, Cd, Cu, Cr, Pb, Hg, Ni, Se). For blanks, use an empty vessel. Each digest contains a blank and blank spike.

2. Add 0.5 ml of H_2SO_4, then microwave at 60% power for 10 minutes or until samples char.

3. Add 15 ml of nitric acid. Swirl.

4. Pre-digest overnight. Cover vessels loosely with lids.

5. Place lid on the teflon vessel and tighten the lid using the capping station to achieve proper torque.

6. Place all vessels (up to 12) in the turntable of the microwave oven, making certain to connect vent tubes to the venting trap.

7. Begin heating the oven: 1) 30% power for 30 minutes; 2) 50% power for 60 minutes; and 3) 70% power for 100 minutes.

8. Remove vessels from the oven, allow to cool to room temperature. A cool water bath can be used to speed the cooling process.

9. Remove the caps using the capping station. Add 2 ml H_2O_2 to samples, set oven at 80% power for 15 minutes. Watch samples closely to be sure they do not char. Add additional HNO_3 if sample is beginning to char and more H_2O_2 if needed to lighten the sample. Increase time if samples digestion is not complete.

10. To test if a digestion is completed, add 1 ml distilled H_2O to the cooled digest. If the sample becomes cloudy, add nitric acid and continue heating followed by addition of hydrogen peroxide until a complete digestion is obtained.

11. Transfer cooled digests to 10 ml volumetric flasks and take to volume with 2% nitric acid. Transfer diluted samples to 30 ml Nalgene bottles for storage until analyzed.

NOTES

1. Acid washing of glass, teflon and plastic can be accomplished by soaking clean glassware in a nitric acid bath containing 4:1 H_2O:concentrated HNO_3 (reagent grade). After acid-soaking, acid washed items should be rinsed in distilled water, dried, and stored in clean, covered cabinets. Protective clothing, gloves, and a full face shield should be worn at all time when working with acid baths.

REFERENCES

1. AOCS. (1978) Analyses for chromium, copper, iron, and nickel in vegetable oils by atomic absorption spectrophotometry. (Ca 15-75). *In* Official and Tentative Methods of the AOCS, third edition, R.O. Walker (ed.), Am. Oil Chem. Soc., Champaign, IL, 4p.

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4. Bock, R. (1979) A Handbook of Decomposition Methods in Analytical Chemistry. John Wiley and Sons, New York.

5. Kingston, H.M. and B. Jassie. (1986) Microwave energy for acid decomposition at elevated temperatures and pressures using biological and botanical samples. Anal. Chem. 58:2534-2541.

6. Aysola, P., P. Anderson, H.L. Cooper. (1987) Wet ashing in biological samples in a microwave oven under pressure using poly(tetrafluoroethylene) vessels. Anal. Chem. 59:1582-1583.

5.2 FLAME ATOMIC ABSORPTION (Fe and Zn)

INTRODUCTION

Flame atomic absorption (AA) spectroscopy is used to analyze Fe and Zn in acid digested fish oil and ester samples. Although flame AA is not the most sensitive method available for these elements, it is the least time consuming and is of adequate sensitivity for the purposes of quality assurance of the biological test materials. This is especially from the standpoint of determining the safety and nutritional composition of the oil. Also, these elements usually occur in concentrations high enough so that flame AA is preferred (due to its lower sensitivity) over graphite furnace.

PRINCIPLE

Metals in the original oil or ester samples are present in the digested sample in their elemental forms. Nitric acid serves to maintain the elements in solution, minimizing binding of the elements with glass in which the samples are diluted or with plastic in which the samples are stored. The basis of flame AA is that each element absorbs light of specific wavelength(s). A hollow cathode lamp is used as a source of light of a specific wavelength. The light is detected by a wavelength specific detector. A sample containing the element of interest is aspirated into a flame located in the path of the light beam. The detector determines what portion of the incident light is absorbed by the sample. A linear relationship exists (Beer's Law) between the concentration of the absorbing element and the attenuation of the light beam. A calibration curve of concentration versus absorbance is created using standards containing each element of interest. The samples, standards, and blanks are then analyzed and concentrations of the element are calculated by linear regression analysis using the calibration curve.

APPARATUS

- Flame atomic absorption spectrophotometer, dual beam, with background correction capability, equipped with an air/acetylene burner head (Perkin-Elmer 5100)
- Acetylene, atomic absorption grade
- Compressed breathing air
- Single element hollow cathode lamps Fe and Zn (Perkin-Elmer)
- 25 ml volumetric flasks, glass or polypropylene
- 5 ml, 1 ml, and 0.2 ml automated pipets and tips (Rainin Pipetman or equivalent)
- 5 ml polypropylene test tubes with caps (Sarstedt), acid washed

- Nalgene bottles, 30 ml acid washed
- IBM or compatible computer, monitor (Andek or equivalent), and printer (Star or equivalent)

REAGENTS

- 1000µg/ml metal standard solutions: Fe, and Zn (Fisher or equivalent)
- Ultrex HNO_3, 2% v/v in distilled H_2O (J.T. Baker)

PREPARATION OF STANDARDS & SAMPLES

I. Standards: For each element a 3-point standard curve is prepared by quantitatively diluting commercially prepared 1000µg/ml standard solutions in 2% Ultrex HNO_3. Standards are made up in 25 ml volumetric flasks and transferred to 30 ml acid washed Nalgene bottles for storage. The standard concentrations used depend upon the detection limits and linear working range for each element and the concentration of each element anticipated in the oil or ester samples (Table 5.2-1).

TABLE 5.2-1. Metal Standards For Flame Atomic Absorption.

	ppm Concentration (µg/ml)		
Element	Std 1	Std 2	Std 3
Fe	1.0	2.0	4.0
Zn	0.05	0.15	0.30

II. Samples: Samples must be diluted quantitatively with 2% Ultrex HNO_3 to a concentration which falls within the standard curve designated above. The dilutions necessary are derived empirically.

For aspiration of sample into the flame, disposable 5 ml polypropylene tubes are convenient.

DETERMINATION

Standard parameters used for optimizing the determination of these elements are listed in Table 5.2-2. These parameters are specific to a Perkin-Elmer 5100 spectrophotometer employing single element hollow cathode lamps (1). Conditions may vary for other instruments.

TABLE 5.2-2. Instrument Parameters for Flame Atomic Absorption Analyses of Metals (Perkin-Elmer 5100).

Metal	Wavelength (nm)	Lamp current (ma)	Flame	Slit	(Characteristic Concentration) Sensitivity (0.0044 AU)
Fe	248.3	30	lean	0.2	.039 mg/L
Zn	231.9	15	lean	0.7	.011 mg/L

CALCULATION

Using a microprocessor (computer) based instrument, the data output is mg/L of digested sample. Once the concentration in mg/L of diluted digested sample is obtained, the concentration in the original sample (oil or esters) is calculated as follows:

$$\mu g/g = \frac{(mg/L)(dilution)(total\ L\ in\ digest)}{g\ sample} \times 1000 \left(\frac{1000\ \mu g}{1 mg}\right)$$

PRECISION

The precision of flame atomic absorption spectrophotometry determinations vary according to sample matrix. Generally ashed or acid digested samples of biological origin are precise within 10%.

NOTES

1. All glassware and plasticware must be acid washed. This can be accomplished by soaking clean glassware in a nitric acid bath containing 4:1 H_2O:concentrated HNO_3 (reagent grade). After acid-soaking, clean items should be rinsed in distilled water, dried, and stored in clean, covered cabinets. Disposable pipet tips are rinsed with 2% nitric acid before use.

REFERENCES

1. Analytical Methods for Atomic Absorption Spectrophotometry. (1982). Perkin Elmer Corp., Norwalk, CT.

5.3 VAPOR GENERATION (Hg)

INTRODUCTION

Cold vapor analysis is utilized for the determination of Hg in microwave HNO_3 digested oil and ester samples. Vapor generation methods were developed for volatile metals for which flame atomic absorption is either insensitive or unreliable. These techniques are standard methods used by the EPA (1). The drawback of hydride generation in the past has been the variability of results between analysts due to the considerable technique required. The BTM Program utilizes an automated vapor generation system which makes the analyses routine and reproducible.

PRINCIPLE

In the mercury cold vapor technique, Hg in acidic medium is reacted with a reducing agent, $SnCl_2$, in a closed vessel for a known length of time. Elemental Hg, which produces a small amount of vapor at room temperature, is swept into the quartz cell. Absorption of light at a specific wavelength is proportional to the amount of Hg present in the cell.

APPARATUS

- Atomic absorption spectrophotometer, dual beam, equipped with background correction (Perkin-Elmer 5100)
- Atomic Vapor Assembly (IL440 Thermo Jarrel Ash or equivalent)
- Electrodeles discharge lamp - Hg (Perkin-Elmer)
- Fleakers, 150 ml and 300 ml with lids
- Stir bars, teflon
- Graduated cylinder, 50 ml
- volumetric flasks, 25 ml
- Computer (IBM or compatible)
- Monitor (Amdek or equivalent)
- Printer (Star or equivalent)

REAGENTS

- HCl, ACS grade (J.T. Baker), 1M and 3M solutions
- $SnCl_2$, ACS grade (Baker)
- Deionized H_2O
- Ultrex HNO_3, 2% v/v in distilled H_2O (Baker)
- Nitrogen
- 1000 µg/ml metal standard solution - Hg

PREPARATION OF STANDARDS AND SAMPLES

1. Standards: Make 3 standards containing 50, 100, and 200 ng Hg in 50 ml 1M HCl. Transfer entire volume of each to a 150 ml fleaker with a stir bar.

2. Samples: Add 2 ml of sample to 50 ml of 1M HCl in 150 ml fleakers with a stir bar.

DETERMINATION

The reducing reagents and instrument settings used for Hg are listed in TABLE 5.3-1 (2). Instrumental settings on the spectrophotometer should be optimized as described in the instrument manual. Peak height integration method is used along with a twelve second integration in an unheated absorption cell.

TABLE 5.3-1. Instrument Parameters For Hydride Generation.

Element	Slit (nm)	Wavelength	Reducing reagent	Reagent setting	Nitrogen Flow (LPM)	Reaction Time (min)
Mercury	0.70	253.6	5% SnCl2 in 25% HCl	5	5-6	1.0

1. **Zeroing the instrument**: Place a "blank" fleaker containing 50 ml 1M HCl in the sample well of the hydride generator. Press "auto zero" on the AA and "run" on the vapor generator. The instrument will automatically flush the sample fleaker with nitrogen, dispense the designated amount of reagent, stir the sample for a designated "reaction time" and purge the head volume again with nitrogen, sweeping any hydrides into the quartz cell for determination of absorption. The AA will automatically "zero" on this sample.

2. **Standard curve**: A standard curve of concentration *vs* absorption is generated by analyzing the 3 concentrations of metal standard prepared above. To analyze each standard, the concentration of the standard is already entered into the microprocessor on the AA as per instructions in the instrument manual, then "run" is pressed on the vapor generator. The instrument will automatically flush the fleaker with nitrogen, dispense the designated amount of reagent, stir the sample for a designated "reaction time" and purge the head volume again with nitrogen, sweeping any hydrides into the quartz cell for determination of absorption. Once all standards have been analyzed, the standard curve stored in the AA microprocessor is used to calculate the concentrations of metals in the samples.

3. **Samples**: Samples in 50 ml 1M HCl are analyzed using the identical procedure as for the standards above.

CALCULATION

The atomic absorption spectrophotometer will generate absorbance values based on peak height of stds. Using blank and stds, a correlation coefficient is created from std curve. Sample absorbance values are converted to total ng amount, then to µg/g.

$$\mu g/g = \frac{total\ ng}{sample\ wt(g)} \div 1000 \left(\frac{1\mu g}{1000ng}\right)$$

PRECISION

Mercury cold vapor techniques are generally precise to within 10%.

REFERENCES

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2. Thermo Jarrel Ash Corp. (1984) Atomic Vapor Accessory Operators and Service Manual. Thermo Instrument Systems, Inc., Waltham, MA. 25p.

5.4 FURNACE ATOMIC ABSORPTION (As, Cd, Cr, Cu, Pb, Ni, Se)

INTRODUCTION

Furnace atomic absorption is used to analyze As, Cd, Cr, Cu, Pb, Ni, and Se in acid digested fish oil and ester samples. Furnace AA sensitivity is greater than flame AA and it is used for ultra-trace analyses (ppb).

PRINCIPLE

The basic principle of the graphite furnace is very similar to flame AA, but the furnace has greater sensitivity and lower detection limits using less sample volume. Matrix modification is used to stabilize the sample as it goes through the furnace program. The 5100 uses Zeeman background correction. Furnace uses electromagnetic radiation which is hotter than the flame. Using an autosampler, the sample is injected onto a platform inside a graphite tube located in the furnace; all in the path of the light beam. The sample absorbance is read at a particular wavelength, specific for each element. A linear relationship exists (Beer's law) between the concentration of the absorbing element and the attenuation of the light beam. A calibration curve of concentration versus absorbance is created using standards containing each element of interest. The samples and blanks are analyzed and concentrations of the elements calculated by linear regression analysis.

APPARATUS

- Atomic absorption spectrophotometer, with Zeeman background correction (Perkin-Elmer 5100).
- Argon gas (UHP)
- Single element hollow cathode lamps - Cd, Cr, Cu, Pb, Ni, and electrodeless discharge lamps - As, Se (Perkin-Elmer)
- 1 and 0.2 ml automatic pipets and tips (Rainin Pipetman or equivalent)
- Furnace Autosampler, AS-60, with tray
- Autosampler sample cups, polystyrene

REAGENTS

- 1000 µg/ml metal standard solutions: As, Cd, Cr, Cu, Pb, Ni, Se
- 2% ultrex HNO_3

- Matrix modifiers - 0.015 mg Pd + 0.01 mg Mg $(NO_3)_2$
  0.20 mg PO_4 + 0.01 mg Mg $(NO_3)_2$
  0.05 mg Mg $(NO_3)_2$

PREPARATION OF STANDARDS AND SAMPLES

I. Standards: For each element a 3-point standard curve is prepared by the autosampler quantitatively diluting commercially prepared 1000 µg/ml standard solutions in 2% Ultrex HNO_3. Standards diluted by the autosampler are made from a 50 ppb or 5 ppb stock solution. The standard concentrations used depend upon detection limits, the linear working range for each element, and the anticipated concentration in the ester or oil sample (TABLE 5.4-1). Appropriate matrix modifiers are used for each specific element.

TABLE 5.4-1. Metal Standards For Furnace Atomic Absorption.

	Concentration µg/L (ppb)		
Element	Standard 1	Standard 2	Standard 3
As	12.5	25.0	50.0
Cd	0.5	1.0	2.0
Cr	5.0	10.0	20.0
Cu	5.0	15.0	30.0
Ni	12.5	25.0	50.0
Pb	12.5	25.0	50.0
Se	12.5	25.0	50.0

II. Samples: Samples are diluted quantitatively with 2% Ultrex HNO_3 to a concentration which falls within the standard curve values designated above. Matrix modifiers are used with each specific element.

DETERMINATION

The standard parameters used to optimize the determination of each element are listed in Table 5.4-2. These parameters are specific to the Perkin-Elmer 5100 spectrophotometer (with Zeeman) using single element hollow cathode lamps and electrodeless discharge lamps. Conditions may vary for other instruments. (1,2)

Table 5.4-2. INSTRUMENT PARAMETERS FOR FURNACE ATOMIC ABSORPTION ANALYSES OF METALS

			Temperature (Recommended)				
Element	**Wavelength**	**Lamp current (MA)**	**Pyrolysis**	**Atomization**	**Slit**	**Characteristic Mass (Sensitivity)**	**Matrix Modifiers**
As	193.7	8	1300	2100	0.7	15 pg	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$
Cd	228.8	6	850	1650	0.7	0.5 pg	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$
Cr	357.9	25	1650	2500	0.7	3.3 pg	0.05 mg $Mg(NO_3)_2$
Cu	324.8	15	1300	2500	0.7	8 pg	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$
Pb	283.3	10	850	1800	0.7	12 pg	0.02 mg PO_4 + 0.01 mg $Mg(NO_3)_2$
Ni	232.0	25	1400	2500	0.2	13 pg	No modifier
Se	196.0	5	1100	2100	2.0	28 pg	0.015 mg Pd + 0.01 mg $Mg(NO_3)_2$

CALCULATION

Using a computer based instrument, the data output is µg/L of digested sample. Once the concentration in µg/L of the diluted sample is obtained, the concentration in the original sample oil or ester is calculated as follows:

$$\mu g/g = \frac{(\mu g/L)\ (dilution)\ (total\ L\ in\ digest)}{sample\ wt.\ (g)}$$

REFERENCES

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6.0 MOISTURE

INTRODUCTION

The AOAC has published a final action Karl Fischer (KF) method (#28.003-28.005) (1) for the determination of moisture in oils and esters. A collaborative study on the determination of water in oils and fats using the KF method has been reported (2). The method employed by the BTM Program is a modification of the AOAC method for use with a pyridine-free KF reagent and a Mettler DL-18 automated titrator (3,4).

PRINCIPLE

Fish oil or esters of fish oil are dissolved in chloroform-methanol (1:1) and titrated with the KF reagent to an electrometric end-point. Water reacts stoichiometrically with iodine in the presence of SO_2, methanol, and a suitable base (RN) in the following reaction:

$$H_2O + I_2 + 3RN + SO_2 + CH_3OH \rightarrow (RNH)SO_4CH_3 + 2(RNH)I$$

A single component system is used in which the KF reagent contains I_2, SO_2, and base. Methanol is present as a solvent. The titration is followed using a two pin platinum electrode which has a current source applied to its poles. The voltage at the electrode pins is the input signal measured. When the last traces of H_20 have been titrated the voltage drops to virtually zero which signals the electrometric endpoint.

APPARATUS

- Karl Fischer titrator (Mettler DL-18 or equivalent)
- Burette, 10 ml (Mettler or equivalent)
- Printer (Epson FX-85 or equivalent)
- Balance, 4 place, with RS232 interface (Mettler AE200 or equivalent)
- Glass titration vessel, 150 ml (Mettler or equivalent)
- Wide bore syringe (10 ml capacity)
- 1 ml syringe
- Automatic pipet

REAGENTS

- Hydranal - Composite 5, pyridine free (5 mg H_20/ml) (Reidel-deHaen)
- Chloroform:methanol (1:1) (ACS grade)
- 3M KCl/sat AgCl reference electrode solution

PROCEDURE

Approximately 2 g samples are used for oils and esters. Approximately 0.2 g samples are used for determination of moisture in ethanol. No sample preparation of the ethanol, oils or esters is necessary since they are freely soluble in chloroform:methanol.

TABLE 6.0-1. Autotitriator Configuration for Moisture Analysis.

Configuration Number	Value	Explanation
1	15	switch-off delay (seconds)
2	1	report in %
3	1	pyridine-free single component KF reagent
4	0	calibration sample: water
5	1	automatic pretitration before each run
6	9999	request stir time for each sample
7	0	no blank value
8	0	requests no sample number
9	2	acoustical signal at end of titration
A	6	max vol per titration: 6 burette strokes

1. Configure the autotitrator with the settings listed in TABLE 6.0-1.

2. Determine concentration of the KF reagent (Hydranal) using water as the standard. Pipette 50µl water into a tared titration beaker, using a 200µl pipettor. The weight will automatically be entered into the microprocessor from the balance. The result is stored automatically in memory and is used in the evaluation of titrations that follow. The concentration should read 45-50.

3. Determine the background titrant consumption due to moisture leaking into the titration beaker. A 3-5 minute titration, of pre-titrated solvent, is carried out. The result is stored in the instrument as the "drift" parameter. A value of <1µg/min is acceptable.

4. To determine moisture in sample: dispense 40 ml of solvent (chloroform-methanol, 1:1) into the titration vessel. The "RUN" command will initiate automatic titration of the solvent to dryness. Draw the sample into a wide bore syringe. Place the syringe on a balance. Tare the balance. Inject the oil sample into the titration vessel. Weigh the empty syringe to determine weight of the oil. The weight of the oil is entered into the memory by pressing "RUN" (the negative sign is ignored).

5. The sample is automatically titrated to dryness and background corrections are made. After 2 oil samples, empty the titration vessel, dispense 40 ml of new solvent, and titrate to dryness before analyzing additional samples.

CALCULATION

Water content of the sample is calculated automatically by the autotitrator as percent moisture.

PRECISION

The measuring range of the autotitrator is 10µg-500 mg water, or 1 ppm to 100%. The resolution is 1/2000th of the burette volume. Relative standard deviations are below 10% for low moisture samples and below 2% for high moisture samples.

REFERENCES

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7.0 SENSORY ATTRIBUTES

The BTM Program uses sensory analysis to determine low levels of oxidative degradation of the products and to evaluate the acceptability of BTM products for human consumption. Two sensory tests are performed on both oil and ester concentrates; odor/flavor profile evaluation and color. Odor and flavor profiles are analyzed by a trained sensory panel consisting of five to six individuals. The methods used for sensory training are presented in section 7.1. Color is analyzed by the Gardner method, using a standard scale for color. Sensory analyses have proven to be as sensitive or more sensitive than many of the instrumental methods available.

7.1 SENSORY TRAINING; TRIANGLE TEST AND INTENSITY RANKING

INTRODUCTION

Prospective sensory panelists are trained using standard methodology (1,2). Panelists are first screened for flavor and odor sensitivity using the triangle difference test for the four basic tastes: sweet, sour, salty, and bitter. Once the panel is selected, the individuals are trained to identify specific flavor and odor components, using a series of chemical standards (3,4). An unstructured scale (5) is used to measure weak versus strong intensity of the odor and flavor components identified by chemical standards. Finally, the responses of the individuals on the taste panel are "calibrated" to total odor/flavor intensity using fish oil standards.

PRINCIPLE

The triangle difference test is a standard test used to determine whether the panelist possess sufficiently sensitive taste and odor discrimination (1,2). Once a person is selected for the panel, they undergo sensory training, which utilizes chemical standards and known fish oil standards. This training helps to minimize the subjective responses of individual panelist. A consensus is reached among panelists on the definition of odor/flavor descriptors using chemical standards. Known fish oil standards are then used to reach a consensus on the intensity rating which will be assigned to a standard sample. This is to calibrate the panelists' responses to unknown samples.

APPARATUS

- Volumetric flasks, 100 ml
- Analytical balance, capable of weighing to four decimal places
- Beakers, 40 ml
- Watch glasses to fit 40 ml beakers
- Waterbath, 50°C

REAGENTS

- Bitter — Caffeine, 0.035%, 0.07% 0.14%
- Sweet — Sucrose, 1%, 2%, 4%
- Salty — NaCl, 0.1%, 0.2%, 0.4%

• Sour	Citric acid, 0.035%, 0.07%, 0.14%
• Buttery	H and R artificial butter flavor, 0.0005%, 0.001%, 0.005%
• Beany	soybean oil toasted soybeans
• Painty	edible linseed oil heated for 4, 6, 8, and 10 days at 60°C
• Stale/Oxidized	soybean oil heated 10 days at 60°C *t*-2-*t*-4-decadienal, 50μg/ml cottonseed oil heated 15 days at 60°C *c*-4-heptenal, 5μg/ml
• Grassy	*c*-3 hexenol 500μg/ml, 200μg/ml *t*-2-*c*-6-nonadienal, 2μg/ml, 10*u*g/ml *t*-2-hexenal, 30μg/ml
• Raw Green	olive pits
• Fishy	trimethylamine HCl, 50μg/ml cod liver oil
• Solvent	ethanol, isopropanol
• Fruity/Perfumey	olive oil esters honeydew melon, grapes
• Liquor	almond liqueur, wine
• Burnt	scorched sugar
• Musty	raw potato, fresh earth
• Decomposition	unprocessed fish oil
• Characteristic protein flavor (CPF)	washed fish mince
• Fish oil reference sample	(Note 1)

DETERMINATION

I. Triangle test

1. Prepare 100 to 500 ml of each standard for the four basic flavor components (sweet, salty, sour, and bitter) at the three specified concentrations.

2. Aliquot 20 ml of each of the standard solutions into 50 ml beakers.

3. Present each prospective panelist with three samples, two of which are the same and one which is different, beginning with the lowest concentration.

4. The prospective panelist is required to pick the sample believed to be different.

5. Repeat this test for twelve combinations to test for each of the four basic tastes at three concentrations.

6. The prospective panelist must be correct on at least 60% of the responses in order to qualify for the panel.

II. Training for specific odor components

1. Provide panelist with the array of standards listed under "reagents". The standards should be at room temperature and clearly labeled for the flavor attribute they represent.

2. Panelist familiarize themselves with the odors as they are defined by the standards provided.

3. Place a 20 ml fish oil sample in a 40 ml beaker, cover with a watch glass, and heat for not less than 10 min or more than 40 min in a 50°C water bath.

4. The panelist remove the beaker from the water bath, dry off any adhering water drops, remove the watch glass, inhale the vapors above the oil sample and identify the odor components present, using the chemical standards to define them.

5. After each panelist has identified odor components present, the panel discusses the results and reaches a concurrence on what odor components are present.

6. The "standard" fish oil sample (stored frozen at -30°C) is analyzed simultaneously with all samples evaluated for odor and flavor profile.

III. Calibration of the unstructured scale

1. An unstructured scale is used (Figure 7.1-1) on which total intensity of flavor, total intensity of odor and each flavor/odor component may be ranked from "absent" to "strong". The scale is 15 cm in length. In order to rank an oil, the

panelist marks a slash across the scale at the point believed to best rate each attribute.

2. The "standard" fish oil is used to calibrate the scale near the "absent" end of the scale. Using a "strong" oil sample or reference oil specially created for this purpose (a thermally abused oil sample may be used), the scale is then calibrated at the "very strong" end of the scale. Each panelist is asked to mark a response on the scale. The responses of all panel members are then compared and a consensus reached on how to respond to a sample of this intensity.

3. The same samples are given in two subsequent taste panel sessions to test the calibrations of the panelist, and any outlying responses are corrected.

FIGURE 7.1-1. Odor/Flavor Profile Score Sheet

SENSORY ANALYSIS OF BIOMEDICAL TEST MATERIALS

DATE: _____

PANELIST: _____

SAMPLE: _____

Place a vertical mark across the holzontal line according to your judgment as to the intensity of each attribute. Mark only the attributes you detect. Please label the "INTENSITY" line with either an "O" (odor) or "F" (flavor). The line is 15 cm. long.

ABSENT VERY STRONG

TOTAL INTENSITY - Odor and Flavor (Use line below by entering point with "O" for odor and "F" for flavor.

|—————————————————————————————————————|

BUTTERY

|—————————————————————————————————————|

BEANY (soy or other raw bean)

|—————————————————————————————————————|

ACRID (throat-burning)

|—————————————————————————————————————|

SOAPY

|—————————————————————————————————————|

PAINTY (linseed oil or drying paint)

|—————————————————————————————————————|

STALE (old cooking oil)

|—————————————————————————————————————|

GRASSY (freshly cut grass)

|—————————————————————————————————————|

RAW GREEN (olive pit)

|—————————————————————————————————————|

FISHY (cod-liver oil)

|—————————————————————————————————————|

BITTER (caffeine or quinine)

|—————————————————————————————————————|

SOLVENT (chemical)

|—————————————————————————————————————|

FRUITY/PERFUMEY

|—————————————————————————————————————|

LIQUOR (almond, rum, wine, brandy)

|—————————————————————————————————————|

SWEET

|—————————————————————————————————————|

BURNT (carmelized/scorched sugar)

|—————————————————————————————————————|

MUSTY (earthy)

|—————————————————————————————————————|

DECOMPOSITION (decay)

|—————————————————————————————————————|

CPF (wet dog)

|—————————————————————————————————————|

OTHER (_____)

|—————————————————————————————————————|

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7.2 ODOR AND FLAVOR PROFILE EVALUATION

INTRODUCTION

The odor and flavor profiles are used by the BTM Program to evaluate the fish oil and ester products destined for human consumption. In addition, the evaluation can detect low level oxidative degradation not easily detected by instrumental methods. A trained sensory panel, consisting of five to six individuals, is used to evaluate the total and individual intensities of odor and flavor of the product. The odor and flavor intensity of eighteen individual flavor components along with a total overall intensity are evaluated using an unstructured scale. The unstructured scale is a modification of the Quantitative Description Analysis method (QDA) (1,2).

PRINCIPLE

Fish oil or esters of fish oil (approximately 5 ml) are heated for a known length of time (10 min) in a water bath at 50°C in a beaker covered with a watch glass. Heating the sample makes volatile components more available for odor and flavor evaluation. The sensory panelist remove the beaker from the water bath, wipe off any adhering water drops, remove the watch glass, and inhale the vapors in the head space above the oil sample. Each panelist then evaluates the sample for total intensity of odor and for the intensity of eighteen different odor characteristics (3-9) frequently used in the analysis of oils: buttery, beany, acrid, soapy, painty, stale/oxidized, grassy, raw green, fishy, bitter, solvent, fruity/perfumey, liquor, sweet, burnt, musty, decompositon, and CPF. Intensity is recorded on an unstructured scale (15 cm in length) that ranges from "absent" to "strong" (Figure 7.1-1). The panelist marks a vertical line at a point along the unstructured scale which best reflects the intensity of the attributes detected. The panelist then take approximately 50-100µl of sample into his/her mouths to analyze, first for total intensity of flavor, and then for identification and quantification of intensity of the same eighteen flavor components. The flavor/odor components identified have previously been defined by a consensus of the panel during the training sessions. For the purposes of this program, the panelists agreed upon the definitions listed in TABLE 7.2-1.

TABLE 7.2-1. Flavor/Odor Definations.

BUTTERY	aroma and flavor of sweet, freshly-churned butter. A strong buttery flavor refers to a pronounced sweet butter flavor, not old or rancid.
BEANY	characteristic of soybean oil products or fresh raw soybeans, or other raw beans.
ACRID	throat-burning,
SOAPY	having qualities of soap; more of a mouth feel; slick or slightly foamy.
PAINTY	resembling drying paint or strong linseed oil.
STALE/OXIDIZED	sharp, old cooking oil; characteristic of oils exposed to air, particularly cottonseed oil. A strong oxidized flavor is termed "rancid".
GRASSY	"green" flavor or odor of freshly cut grass; slightly characteristic of cucumbers.
RAW GREEN	olive pits.
FISHY	characteristic odor and flavor of fish oil, such as cod-liver oil.
BITTER	taste and odor of caffeine or quinine; may be acrid.
SOLVENT	alcohol odor or taste.
FRUITY/PERFUMEY	fruity or floral; may be slightly citrus or having the characteristics of honeydew melon; characteristic odor and flavor of fish oil esters.
LIQUOR	almond liqueur, rum, wine, brandy
SWEET	pleasant, sucrose odor and flavor; slightly characteristic of syrup.
BURNT	scorched; like caramelized sugar; may be found in conjunction with sweetness.
MUSTY	earthy; raw potato or fresh earth.
DECOMPOSITION	decay, amine-like
CPF	characteristic protein flavor; washed fish mince

The advantage of the unstructured scale over an interval scale is that the panelist do not have to fit their perceptions into specifically designated categories. Further, the open line scale generates data that can be analyzed by standard statistical methods which assume a normal distribution (1).

Because a panelist's response may vary between days and by time of day, each analysis is accompanied by the analysis of a standard oil which serves to calibrate their responses. In order to avoid sensory fatigue, no more than three samples are analyzed in one session. For QA purposes, each sample is analyzed by the panel in two separate sessions and the mean of the two analyses is reported.

APPARATUS

- Beakers, 40 ml
- Watch glasses
- Water bath, 50°C
- Disposable dispensing pipets
- Score sheets
- Warm water
- Paper cups

REAGENTS

- Fish oil reference standard (Note 1)

DETERMINATION

1. Oil samples and the oil standard (5-6 ml) are placed in glass beakers, covered with watch glasses and preheated in a 50°C H_2O bath for 10 minutes prior to convening the panel.

2. The panelist removes each beaker from the water bath, wipes off adhering waterdrops, removes the watch glass and inhales the vapors above the sample. Each sample is rated for total intensity of odor and for the presence of the 18 odor components listed on the score sheet. Intensity is recorded on an unstructured scale (15 cm in length) that ranges from "absent" to "strong". The panelist marks a vertical line at a point along the unstructured scale which best reflects the intensity of the sample component on the score sheet.

3. Samples are then tasted in the order of increasing odor (sample with the least odor is tasted first) using disposable dispensing pipets to pick up an aliquot (50-100µl) of the oil sample. The panelist takes the sample into the mouth, holds the sample on the back of the tongue, draws air into the mouth and exhales through the nose. The panelist records the flavors detected and their intensity on the score sheet.

4. The panelist rinse his/her mouth with warm H_20 between samples.

CALCULATION

1. The scores are calculated for each flavor component by measuring the distance along the 15 cm scale at which the panelist placed a vertical mark. The scores are recorded to the nearest 0.1 cm.

2. A mean and standard deviation are calculated for each odor/flavor component and for the total intensity of odor and flavor for each panel session. For QA purposes, an overall mean and standard deviation is calculated from all panels that evaluated a particular product.

PRECISION

The "standard" oil is evaluated at each sensory panel. During panelist training it was agreed that the standard oil would be considered to possess a value of 2.0 on the 15 cm scale. A random selection of 20 sensory panels conducted at the Charleston Laboratory yielded the following means and standard deviations for the "standard oil" *between panel sessions*: total intensity of odor, 1.68 $\pm$ 0.29 (17.5% RSD); total intensity of flavor, 1.74 $\pm$ 0.43 (24.6% RSD). The average deviations between panelists over the same twenty sessions was 1.68 $\pm$ 0.56 for total intensity of odor and 1.74 $\pm$ 0.75 for total intensity of flavor.

NOTES

1. A mild steam-stripped fish oil is used as a reference standard. It was decided by the panel that the reference standard would be rated at approximately 2 cm from the left end of the scale for "total intensity of odor" and "total intensity of flavor". This standard is evaluated with all samples. The samples are compared to the reference standard and rated accordingly. Tubes containing aliquots of the reference standard are stored in the freezer at -30°C and used as needed.

REFERENCES

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7.3 COLOR DETERMINATION/GARDNER METHOD

INTRODUCTION

The color of fish oil and esters of fish oil is analyzed as part of the QA/QC process in the BTM Program using the standard Gardner method (1). Color can greatly affect human appeal for test products, especially when compared with placebo oils and may potentially influence the conduct of double blind studies. Color can also act as an indicator of oxidative change during processing and storage.

PRINCIPLE

The color of fish oil is determined by comparison with that of a range of 18 consecutively numbered standards of specified color, using a colorimeter (1-3). The standards used in this method are called Hellige color discs. The number of the color standard which most closely matches the sample is recorded as the Gardner number for that particular sample.

APPARATUS

- Hellige color comparator (Hellige Inc., Garden City, NY)
- Color standards discs for 1933 Gardner color scale, Nos. 620C-40 and 620C-42 (Hellige Inc., Garden City, NY)

REAGENTS

- Distilled water

PREPARATION OF STANDARDS & SAMPLES

1. The standards require no preparation.
2. The sample is determined to be clean and free of particulate matter. This may be accomplished by filtering.

DETERMINATION

1. Place 5-10 ml of sample in 12 x 115 mm glass test tube provided with the color comparator.
2. Place 5-10 ml of distilled water in the reference test tube.

3. Insert the color standard disc in the wheel of the color comparator and rotate until the color standard which most closely matches the oil sample is found.

4. Record the color standard number.

CALCULATION

The number of the color standard which most closely matches the oil sample is recorded as the Gardner Number.

REFERENCES

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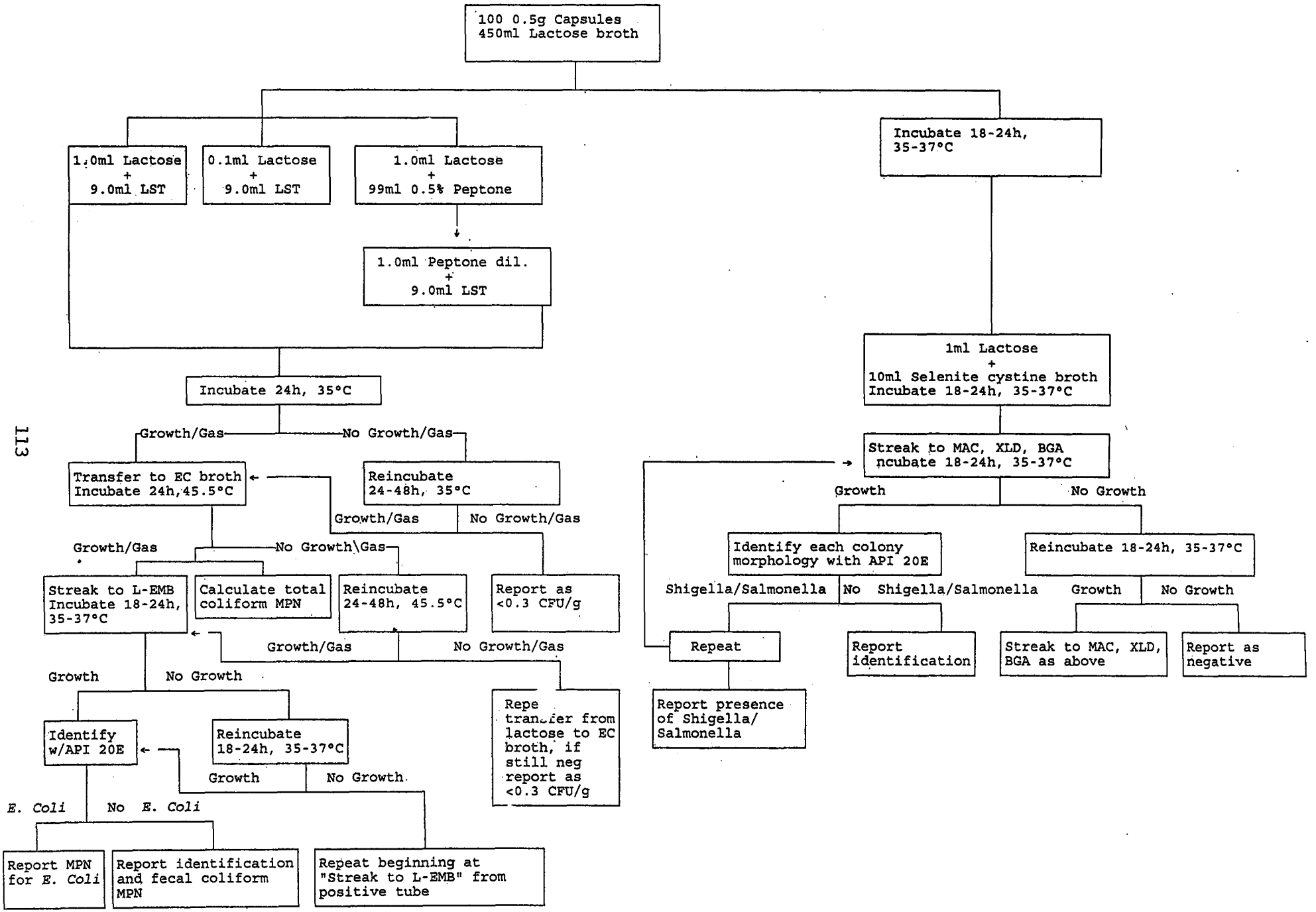
8.0 BACTERIA

INTRODUCTION

Steam- and vacuum-deodorized menhaden oil, placebo oils, and ester concentrates, produced by the BTM Program, may be encapsulated in soft gelatin. This encapsulating material is analyzed for pathogenic coliform bacteria, *E. coli* and *Salmonella*. The U.S. Pharmacopeia specifies that the total bacterial count in gelatin must not exceed 1000 colony forming units per gram (CFU/g), and tests for *Salmonella* and *E. coli* must be negative (1). Bulk oils and esters are not tested for bacteria since the deodorization process kills any bacteria present in the crude oil. The flow chart for the microbiological examination is given on the following page.

FIGURE 8.0-1. Flow Chart for the Microbiological Examination of Gelatin Encapsulating Material.



8.1 COLIFORM BACTERIA

INTRODUCTION

Coliforms are determined using a presumptive multiple tube fermentation test adopted by the AOAC (2) and the FDA (3). The presumptive test is followed by confirmed tests for *E. coli*.

PRINCIPLE

Coliform bacteria are a taxonomically heterogeneous group that are identified by the production of CO_2 from lactose within 48 hr at 35°C. The presumptive multiple tube fermentation test uses the principle of dilution to extinction to estimate the number of bacteria in the sample. Decimal dilutions of the sample are introduced into replicate tubes containing lauryl tryptose (LST) broth. The maximum dilution at which growth occurs theoretically represents the volume containing a single organism. Results are expressed as the most probable number (MPN) of bacteria per gram of sample, which is determined using an MPN probability table.

The confirmed test for total coliforms is based on the ability of coliforms to produce CO_2 in lauryl tryptose broth in 48 hr at 35°C. The confirmed test for *E. coli* is based on the ability of *E. coli* to grow and produce CO_2 at elevated temperature (45°C) after 24 hr in Escherichia Coli (EC) broth.

APPARATUS

- Incubator, 35 ± 1°C
- Covered water bath, 45.5 ± 0.2°C
- Immersion type thermometer, 0-100°C
- Sterile media bottles, Pyrex, 500 ml
- Sterile graduated pipets, 1, 5, and 10 ml
- Sterile utensils for sample handling (forceps, 10cc syringes with needles)
- Glass dilution bottles, 200 ml
- Culture tubes, 16 mm x 150 mm
- Gas tubes, 6 mm x 50 mm
- Sterile petri plates, 100 mm x 15 mm

- Inoculating loop

REAGENTS

- Lactose broth (Difco)
- Lauryl tryptose broth (Difco), (also called lauryl sulfate tryptone brothor LST)
- EC broth (Difco)
- Levine's eosin-methylene blue agar (L-EMB) (Difco)
- Peptone water (Difco)
- Analytab Products, Inc. (API) 20E Strips

SAMPLING

Lots are sampled according to a sampling plan found in Title 50 of the Code of Federal Regulations, Chapter II, Subchapter G, Part 260, Table 3. Under this sampling plan, three sample are randomly drawn from lots containing up to 5,400 containers that are each 12 ounces or less. Six samples are randomly drawn from lots containing 5,401 to 21,600 - 12 ounce containers, and lots with 21,601 to 62,400 containers require 13 samples. At the Charleston Laboratory, bottles of encapsulated oils are each 100 g and placed in master cartons containing 24 bottles. There are, therefore, subject to this sampling protocol. The sampling plan calls for the analysis of the entire contents of each container.

REMOVING OIL FROM CAPSULES

Place a sterile pad (absorbant paper maybe wrapped in BioShield II Sterile Wraps and autoclaved) under a laminar flow hood. Place a bottle of capsules under the hood, open, and remove capsules with sterile forceps. Hold capsules with sterile forceps on the sterile pad and withdraw all of the oil from each, using a sterile needle and syringe. Place 100 empty capsules into a sterile (500 ml) media bottle. Discard the oil in waste container designated "Fish Oil Waste", located in the production facility. Use a new needle and syringe for each bottle of capsules or when the needle dulls. A new needle and syringe must be used when a new bottle is opened in order to prevent cross contamination of samples.

PREPARATION OF SAMPLES

Standard procedures for the analysis of encapsulated fish and other oils (esters) do not exist. However, semistandardized procedures appear to be in general use to screen capsules for *E. coli*. The following procedure is a slight modification of the procedure used by the encapsulator (and is currently used at the Charleston Laboratory) to screen caspules for *E. coli*.

1. The drained capsules are added to 450 ml of warm (35°C) sterile lactose broth and the broth is incubated at 35 - 37°C for up to 2 hr to dissolve the capsules. The broth is swirled approx. every 10 minutes during the incubation period to facilitate capsule dissolution. This bottle of capsules dissolved in lactose broth will be used for both coliform and *Salmonella* identification procedures.

2. When all capsules have dissolved, add 1.0 ml of the lactose broth to triplicate MPN tubes, each containing 9 ml of lauryl sulfate tryptose broth (LST) and inverted gas tubes. These are labelled 10^{-1}.

3. Inoculate triplicate tubes of LST (9 ml each tube) with 0.1 ml of lactose broth labelled 10^{-2}.

4. Prepare a final dilution by inoculating 1.0 ml of lactose broth into a dilution bottle containing 99 ml of 0.5% peptone buffer, shaking 25 times in 7 seconds in a 1 foot arc. Add 1.0 ml of this dilution to each of triplicate tubes of LST (9 ml each tube) labelled 10^{-3}.

5. Incubate lactose broth for 24 h at 35 - 37°C for *Salmonella* identification procedure.

DETERMINATION

I. Presumptive Test

1. Incubate each LST tube at 35°C for 24 hr. Check tubes for growth and gas (i.e., a gas bubble in the fermentation vial or production of gas when tube is gently agitated). Reincubate negative tubes for an additional 48 hr. Examine 48/72 hr LST tubes for gas production.

2. Perform confirmed tests on all positive tubes (II below).

II. **Confirmed Test for *E. coli***

1. Transfer loopful of LST suspension from positive LST tubes in the presumptive test to a tube containing 10 ml of EC broth and an inverted gas tube. Incubate EC tubes for 24 hr at 45.5°C in a circulating water bath. Examine 48/72 hr LST tubes and transfer positive tubes to EC broth as above. After 24 hr and 48 hr incubation, examine all EC tubes for growth and gas production.

2. From each positive tube of EC broth streak a loopful of suspension to an L-EMB agar plate. Incubate 18-24 hr at 35°C.

3. If there is no growth on the L-EMB plate after 18-24 h, reincubate for another 18-24h. If there is still no growth after the 36-48h total incubation, streak a new L-EMB plate from the EC broth.

4. Select one of each colony morphology and identify biochemically using the API 20E test kit. Prepare a bacterial suspension and set up the API according to the kit instructions. Streak a tryptic soy agar plate with a loopful of suspension as a purity plate. Read the API after 18 - 24 hr. Reread the API after 48 hr if necessary (see kit instructions). Be sure the purity plate confirms the purity of the culture. Record the biochemical profile number on the worksheet provided with the API kit and find it in the Biochemical Profile Index. Record identification.

CALCULATION

Results are calculated from Part I and Part II as the Most Probable Number (MPN) of total coliforms and *E. coli* per gram of capsule material. The following table provides an example of how to record data for calculation of the MPN (3). The combination of positive tubes recorded from each dilution is then applied to a MPN probability table (Note 1).

TABLE 8.1-1. Sample Data Record for Calculation of MPN Coliforms

Sample	Dilution			Combination of Positive Tubes	MPN Index per g (from TABLE 8.1-20)
	10^1	10^2	10^3		
1	3/3	3/3	1/3	3-3-1	4.6
2	3/3	2/3	1/3	3-2-1	1.5
3	2/3	1/3	0/3	2-1-0	.15

PRECISION

The MPN method is an estimate of the density of colony forming units (CFU) in a sample. For high density populations, the MPN is not as precise as a direct plate count. It is most precise for low levels of organisms (<10 per g). For the purposes of quality assurance of biomedical test materials, however, the presence of any *E. coli* results in rejection of the "lot" of material. The limit of detection with a 10 g sample is approximately 1 CFU/g.

NOTES

1. MPN index and 95% confidence limits for various combinations of positive results when three tubes and three dilutions are used are listed in TABLE 8.1-2 (4).

TABLE 8.1-2. Most Probable Numbers Table.

Combination of Positives	MPN index per g	95% confidence limit upper	lower
0-0-0	<0.03	<0.005	<0.09
0-0-1	0.03	<0.005	0.09
0-1-0	0.03	<0.005	0.13
0-2-0	--	--	--
1-0-0	0.04	<0.005	0.20
1-0-1	0.07	0.01	0.21
1-1-0	0.07	0.01	0.23
1-1-1	0.11	0.03	0.36
1-2-0	0.11	0.03	0.36
2-0-0	0.09	0.01	0.36
2-0-1	0.14	0.03	0.37
2-1-0	0.15	0.03	0.44
2-1-1	0.2	0.07	0.89
2-2-0	0.21	0.04	0.47
2-2-1	0.28	0.10	1.50
2-3-0	--	--	--
3-0-0	0.23	0.04	1.20
3-0-1	0.39	0.07	1.30
3-0-2	0.64	0.15	3.80
3-1-0	0.43	0.07	2.10
3-1-1	0.75	0.14	2.30
3-1-2	1.20	0.30	3.80
3-2-0	0.93	0.15	3.80
3-2-1	1.5	0.30	4.40
3-2-2	2.1	0.35	4.70
3-3-0	2.4	0.36	13.0
3-3-1	4.6	0.71	24.0
3-3-2	11.0	1.50	48.0
3-3-3	>11.0	>1.50	>48.0

REFERENCES

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3. Mehlman, I.J., W.H. Andrews, B.A. Wentz. (1984) Coliform bacteria, *In* Bacteriological Analytical Manual, 6th edition. US FDA, Assoc. Ofc. Anal. Chem., Arlington, VA, pp. 5.01-5.07.

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8.2 SALMONELLA

INTRODUCTION

An AOAC approved method employing selective enrichment and selective plating is used to detect the presence of *Salmonella* in encapsulated products (1). This is the same method used by the U.S. Food and Drug Administration (2). In the test, selective plating is followed by confirmation using a commercially available biochemical test kit. Presence of any confirmed *Salmonella* colonies in a sample test material would necessitate rejection of the sample lot for human consumption, as prescribed by the U.S. Pharmacopeia (3).

PRINCIPLE

The methodology for the isolation and identification of *Salmonella* consists of five basic steps: (1) pre-enrichment in a non-selective medium (lactose broth), (2) selective enrichment in a medium which promotes the growth of *Salmonella* while restricting proliferation of most other bacteria (selenite cystine broth), (3) selective plating on brilliant green (BG) agar, MacConkey's (MAC) agar and xylose lysine desoxycholate (XLD) agar, (4) identification of suspicious colonies based on colony morphology, and (5) confirmed biochemical identification using API-20E test kit.

APPARATUS

- Incubator, 35°C
- Sterile media bottles, 500 ml
- Sterile culture tubes, 16 mm x 150 mm
- Sterile culture dishes, 100 mm x 15 mm
- Sterile graduated pipets, 5 and 10 ml
- Innoculating loop

REAGENTS

- Lactose broth (Difco)
- Selenite cysteine broth (Difco)
- Brilliant Green agar (BGA) (Difco)

- MacConkey (MAC) agar (Difco)
- Xylose lysine desoxycholate (XLD) agar (Difco)
- Analytab Products Inc. (API) 20E strips

DETERMINATION

1. Begin with step 5 under Preparation of Samples. Transfer 1 ml of incubated sample from lactose broth to 10 ml selenite cysteine broth.
2. Incubate 18 - 24 hr at 35- 37°C.
3. Streak a loopful of the incubated selenite cysteine broth onto each MAC, XLD, and BGA.
4. Incubate plates 18 - 24 hr at 35 - 37°C.
5. Examine plates for suspect *Salmonella* colonies (Note 1). Record colonial morphologies. Identify any isolate growth on the MAC, XLD, and BGA plates.
6. Pick one isolate of each morphology and identify with the API 20E strips and the biochemcial profile index according to the manufacturer's instructions. Also, be sure to use TSA purity plates as in *E. coli* identification (Sect. 8.1)
7. Reincubate any negative plates for an additional 24 hr and then examine for growth. If plates are negative after a total of 48 h, discard them.

CALCULATION

Presence of any confirmed *Salmonella* colonies in the sample requires retesting of the sample through the entire procedure. Presence of confirmed *Salmonella* in the sample a second time requires that the analysis be carried out on another sample from that product lot. Presence of confirmed *Salmonella* in a second sample from that lot requires that the lot of product from which the samples were derived be rejected for use for human consumption.

PRECISION

This assay tests for the presence of *Salmonella*, but does not provide an estimate of bacterial concentration. Since presence of any *Salmonella* positive colonies necessitates rejection of that sample lot for human consumption, a concentration is not required. The limit of detection using a 10 g sample is approximately 1 colony forming unit per gram.

NOTES

1. Brilliant Green Agar: any blue-green, green, clear or black colonies may represent *Salmonella*, *Shigella*, *Proteus* or some Pseudomonads and must be subjected to biochemical identification.

   XLD agar: Any red, pink, cream or black colony may represent *Salmonella*, *Shigella*, *Arizona*, *Providencia* or *Proteus* species. Not all *Salmonella* species produce black colonies on XLD; therefore, identify any colony that is not a characteristic yellow.

   MAC agar: Any colorless colony (non-lactose fermenter) may represent *Salmonella*, *Shigella*, *Proteus* or Psudomonads and must be subjected to biochemical screening. *E. coli* will be pink to red, usually with a pink precipitate.

REFERENCES

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APPENDIX A

SAMPLING PROTOCOL FOR ENCAPSULATED MATERIAL

Lots are sampled according to a sampling plan found in Title 50 of the Code of Federal Regulations, Chapter II, Subchapter G, Part 260, Table 3. Under this sampling plan, three samples are randomly drawn from lots containing up to 5,400 containers that are each 12 ounces or less. Six samples are randomly drawn from lots containing 5,401 to 21,600 - 12 ounce containers, and lots with 21,601 to 62,400 containers require 13 samples. At the Charleston Laboratory, bottles of encapsulated oils are each 100 g and are placed in master cartons containing 24 bottles. They are therefore, subject to this sampling protocol.

A minimun of 10 capsules is taken from each container. For small lots, additional capsules should be taken from each container to give a total of 100 g of product. The contents of the capsules are pooled in a nitrogen flushed container. This pooled sample is used for all analyses, with the exception of bacteria, to determine lot composition and quality.

In addition to the lot analysis, an individual capsule from each container is analyzed for lipid class and fatty acid composition. Additional samples may be analyzed at the discretion of the QA/QC supervisor.